Title page

University of Alberta

RESPONSES OF VARIOUS NITRATE-REDUCING BACTERIA TO NITRATE AMENDMENT USED TO CONTROL MICROBIALLY-PRODUCED SULFIDE IN OIL FIELD WATERS

by

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Indeed, it is often unrecognized that procaryotes alone could maintain all the nutrient cycles of the earth but that eucaryotes could not. Thus, if all procaryotes were eliminated from the earth, the eucaryotes would soon perish.

Hans G. Schlegel and Botho Bowien, 1987

To
William, Andrew and Clarice

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
CFU	Colony Forming Units
DAP	Dissimilatory Ammonium Production
DAP-NRB	Dissimilatory Ammonium-Producing, Nitrate-Reducing Bacteria
DNB	Denitrifying Bacteria
DNRA	Dissimilatory Nitrate Reduction to Ammonium
FWKO	Free Water Knock Out
GC-MS	Gas Chromatography-Mass Spectrophotometry
HNRB	Heterotrophic Nitrate-Reducing Bacteria
HPLC	High Performance Liquid Chromatography
IC	Ion Chromatography
MPN	Most Probable Number
NRB	Nitrate-Reducing Bacteria
NR-SOB	Nitrate-Reducing, Sulfide-Oxidizing Bacteria
PCR	Polymerase Chain Reaction
RSGP	Reverse Sample Genome Probing
SB1	Serum Bottle #1
SB2	Serum Bottle #2
SRB	Sulfate-Reducing Bacteria
THPS	Tetrakishydroxymethylphosphonium Sulfate
UV detection	Ultraviolet detection

1. Introduction[*]

1.1 General Overview

The presence of H_2S in oil fields can be the result of abiotic or biotic processes. In the later case, sulfate-reducing bacteria (SRB) are the culprits that produce this nocuous gas, leading to "souring" that is defined as the process whereby petroleum reservoirs experience an increase in the production of H_2S during the economic production life of the field (Farquhar 1998). The increase in H_2S content leads to a decrease in the economic value of the gas and oil, as well as operational and health problems associated with the H_2S.

This microbial process in wastewaters and oil field waters can be controlled by another group of microbes, known as nitrate-reducing bacteria (NRB). Their metabolic activities stop sulfate reduction by SRB. In many cases, the NRB can actually consume sulfide, thus decreasing H_2S concentration in the waters. Jenneman et al. (1996) have referred to these sulfide-consuming bacteria as "sulfide bioscavengers". Hitzman and Sperl (1994) used the term "biocompetitive exclusion" to describe the microbial process in which NRB use volatile fatty acids and out-compete SRB. This process prevents or decreases sulfide production and enhances oil recovery.

This chapter reviews: (a) H_2S in the petroleum industry, (b) the metabolism of SRB leading to sulfide production, (c) the occurrence, types and activities of NRB that might be found in oil field waters, (d) some laboratory studies that have elucidated the mechanisms by which NRB control sulfide produced by SRB, (e) some oil field experiences with nitrate injection to control sulfide in wastewaters, surface waters and oil field waters and (f) some of the U.S. patents that apply to this microbial process.

Nitrite addition to control H_2S production in oil fields has been studied (Reinsel et al. 1996, Nemati et al. 2001c). This project has focused primarily on the use of NO_3^- to control sulfide in oil field waters.

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[*] A version of this chapter is being published. Eckford RE and PM Fedorak. (in press). Using nitrate to control microbially-produced hydrogen sulfide in oil field waters. In: Vazquez-Duhalt R and R Quintero (Eds). Petroleum Biotechnology: Development and Perspectives. Elsevier Science.

1.2 H_2S and the petroleum industry

1.2.1 Formation of H_2S

Kerogen is the organic source material from which petroleum is formed and released (Tissot and Welte 1984, Selley 1998). The formation of petroleum occurs in the deeper subsurfaces as burial continues and temperature and pressure increase (Selley 1998). First oil, then gas is expelled from kerogen as the maturation process continues. Significant oil generation occurs between 60 and 120°C, and significant gas generation occurs between 120 and 225°C (Selley 1998). During the maturation process, H_2S is also released. For more information on bacterial and thermochemical sulfate reduction, see Machel (2001).

Machel (2001) wrote, "The association of dissolved sulfate and hydrocarbons are thermodynamically unstable in virtually all diagenetic environments. Hence, redox-reactions occur, whereby sulfate is reduced by hydrocarbons either bacterially (bacterial sulfate reduction) or inorganically (thermochemical sulfate reduction)." Temperature is the major factor determining which process occurs. The microbiological process is common at temperatures from 0 to 60 or 80°C, whereas, the thermochemical process occurs at temperatures greater than 100 to 140°C (Machel 2001). Because temperature increases with burial depth, H_2S found at shallow depths is usually the result of bacterial sulfate reduction, whereas H_2S found at greater depths is the result of thermochemical sulfate reduction (Machel and Foght 2000). However, there are shallow pools that contain higher than expected concentrations of thermochemically generated sulfide (Manzano et al. 1997). These are believed to be the result of thermochemical sulfate reduction occurring downdip and migrating upward to a shallow reservoir (Manzano et al. 1997).

At the time of discovery, the H_2S concentration in an oil field depends upon its maturation history and, or the migration of H_2S into the oil field. However, during oil recovery from some oil fields, an increase in H_2S concentration (souring) can occur as a result of pressurizing the formation by injecting water into the reservoir. This process, know as waterflooding, is discussed in section 1.3. Three well-documented examples of oil field souring are given in the following paragraphs.

Cochrane et al. (1988) describe the souring of the Ninian field in the North Sea. This field was discovered in 1974, and after several years of operation, injection of sea water was used to maintain the production rate. This was followed by an increase in sulfide production attributed to bacterial sulfate reduction. The reservoir temperature was initially between 100 to 120°C, but in the areas adjacent to the injection well bores, the temperature was cooled to as low as 40°C, which was conducive to bacterial sulfate reduction.

Frazer and Bolling (1991) described the souring of the Kuparuk River field on the North Slope of Alaska. The field was initially sweet, but after injection of Beaufort Sea water, detectable levels of H_2S began to appear at the producing wells. The connate water contained essentially no $SO_4^{=}$. However, the $SO_4^{=}$ in the sea water stimulated bacterial sulfate reduction in the reservoir that had a temperature of about 70°C.

The Skjold oil field in the North Sea soured upon the onset of waterflooding (Larsen 2002). Oil and gas production began from this field in 1982, and sea water injection began in April, 1985. In September, 1985, the first recorded H_2S production was measured to be 1.8 ppm in the gas phase. In 2002, the concentrations varied from 10 to 1000 ppm (Larsen 2002). In late 1999, this field produced 1150 kg H_2S d^{-1}.

These examples clearly demonstrate that waterflooding can stimulate bacterial sulfate reduction, leading to souring. Although these examples refer to offshore oil fields, souring also occurs in land-based oil fields using waterflooding (McInerney et al. 1993, Jenneman et al. 1999, Davidova et al. 2001). As a result of the bacterial production of toxic H_2S, the value of the oil decreases as the oil field sours.

1.2.2 H_2S toxicity and properties

H_2S is a very dangerous gas, even though it occurs in nature. Its characteristic rotten egg smell is generally obvious at 0.13 ppm by volume and quite noticeable at 4.6 ppm (American Petroleum Institute 1995). Unfortunately the smell sense becomes quickly fatigued and can fail to warn of higher concentrations. Collapse, coma and death from respiratory failure may occur within a few seconds after one or two inspirations of the undiluted H_2S (Stecher 1972). The U.S. Occupational Safety and Health

Administration has established the acceptable ceiling concentration of 20 ppm (by volume) for H_2S with an acceptable maximum peak above the acceptable ceiling concentration of 50 ppm for an 8-h shift (American Petroleum Institute 1995).

The specific gravity of H_2S is 1.19; therefore, it will collect in low places and accumulate under poorly ventilated conditions (Cord-Ruwisch et al. 1987). H_2S is soluble in water (1 g in 242 mL H_2O at 20°C) and crude oil (Budavari et al. 1996). It is a weak acid existing in aqueous solutions as H_2S, HS^-, or $S^=$ (pKa values of 7.04 and 11.96). Aqueous solutions of H_2S absorb O_2 and form elemental sulfur (Stecher 1972).

1.2.3 Detrimental effects of H_2S

Besides its toxicity, H_2S is a nuisance in the petroleum industry because it contaminates gas and stored oil; corrodes iron in the absence of air (anaerobic corrosion) and precipitates as amorphous ferrous sulfide (FeS), plugging and diminishing the injectivity of water injection wells (Cord-Ruwisch et al. 1987). In addition, fluids with water and H_2S may cause sulfide stress cracking of susceptible metals. This is affected by metal composition, pH, H_2S concentration, total pressure, total tensile stress, temperature and time (Tuttle and Kane 1981).

Two types of cracking, known to occur in wet H_2S environments, are sulfide stress corrosion cracking and hydrogen-induced cracking. The former occurs in steels of relatively high strength and in welds of welded steel structures. A crack propagates under working stress or residual stress vertically to the stress axis (Ikeda and Kowaka 1978). This type of corrosion is most damaging to drillpipe and well production facilities (Bertness et al. 1989). Hydrogen-induced cracking occurs parallel to the surface when no external stress is applied. It is also known as hydrogen blistering because of the blisters that appear on the surface of the metal (Ikeda and Kowaka 1978).

General corrosion attack by H_2S is influenced by the presence of CO_2, O_2 and brine (Cord-Ruwisch et al. 1987, Bertness et al. 1989). It is related to the alloy composition and strength of steel (Bertness et al. 1989). The H_2S forms FeS scale, which is cathodic to the metal, promoting localized attack under the scale and penetration of H_2 into the metal (Bertness et al. 1989, Beech 2002). Figure 1.1 shows the process whereby an anode and cathode pair are generated by the action of SRB acting on sulfates in the presence of iron. The cathode is depolarized as the SRB consume H_2. At the anode, iron (Fe) is oxidized to Fe^{2+} which combines with H_2S produced by the SRB, giving FeS. This process results in a loss of structural material. Heterotrophic SRB also play a role in the deposition of FeS (Figure 1.1).

Removal of dissolved gases (O_2, H_2S and CO_2) from drilling and produced fluids is necessary to minimize corrosion damage. The H_2S in oil base drilling fluid is removed by gas separators and vacuum degassers and then neutralized. Controlling corrosion in H_2S-containing environments requires proper selection of materials, including the use of low-hardness steels, application of inhibitors and complete exclusion and removal of O_2 from water used in petroleum production (Bertness et al. 1989). Clearly, the presence of H_2S greatly increases the cost of exploration for oil and natural gas. It also increases the cost of production and storage of petroleum.

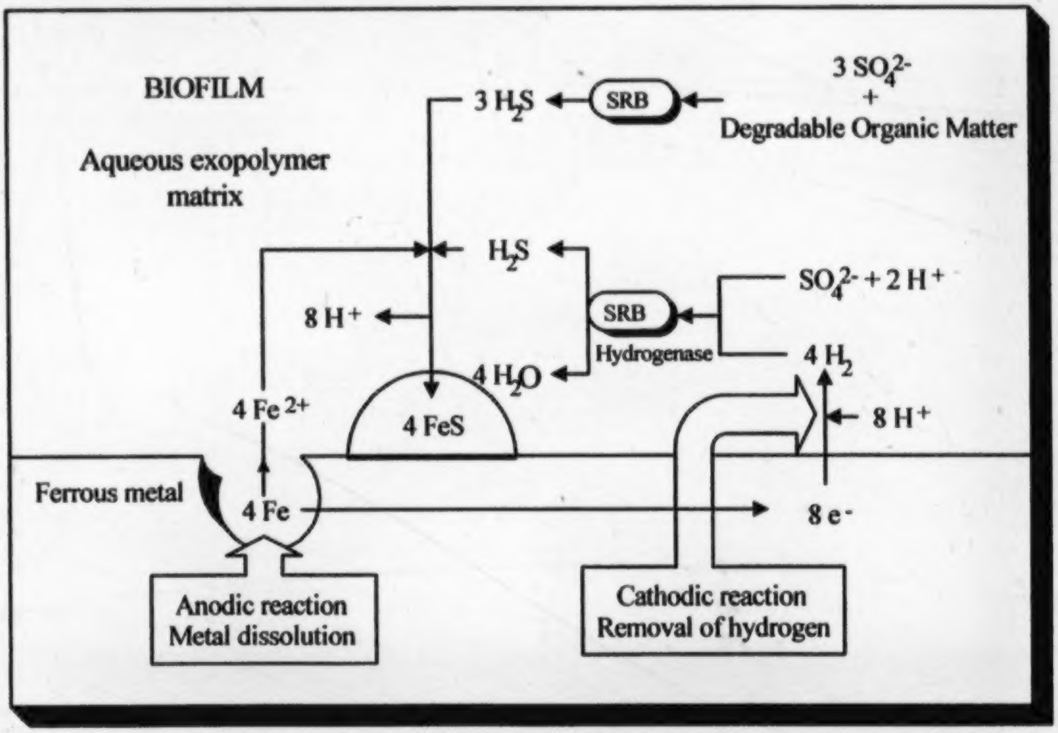
BIOFILM
Aqueous exopolymer matrix
3 H2S
SRB
3 SO42- + Degradable Organic Matter
H2S
SO42- + 2 H+
8 H+
SRB
Hydrogenase
4 H2
4 H2O
4 Fe 2+
4 FeS
8 H+
Ferrous metal
4 Fe
8 e-
Anodic reaction Metal dissolution
Cathodic reaction Removal of hydrogen

Figure 1.1 Iron metal corrosion mediated by SRB in a biofilm. The consumption of H_2 causes cathodic depolarization. Adapted from Cord-Ruwisch (1987).

Plugging (or biofouling) of injection wells is caused by SRB. The sulfide they produce precipitates soluble iron in the injection or formation water and forms colloidal FeS (Iverson and Olson 1984). This colloidal material becomes associated with bacterial cells and oil, forming a gummy mass that can clog reservoirs and plug injection wells. The activities of SRB can also produce calcite ($CaCO_3$) that can add to the plugging problem.

1.3 Oil recovery and waterflooding

Under primary oil recovery, typically less than 30% of the original oil is produced, so that improved or enhanced methods are used to recover some of the remaining oil (Giuliano 1989). These processes, known as secondary and tertiary recovery methods, include the addition of energy into the reservoir and are accomplished by injecting some type of fluid through injection wells. This is referred to as enhanced oil recovery and involves: water injection, gas injection, steam injection, combustion, miscible fluid displacement and polymer injection (Giuliano 1989). In this thesis, only water injection or waterflooding will be discussed.

Waterflooding involves pumping water into the reservoir to stimulate production. The injected water provides pressure to force the oil out of the rock and to sweep it toward producing wells as shown in Figure 1.2. Waterflooding has been attempted in almost every type of reservoir, with its greatest success in relatively homogenous reservoirs having sufficient permeability to allow water injection at a reasonable rate (Giuliano 1989). Up to 60% of the oil can be recovered with waterflooding (Selley 1998). Water handling can become a major operational procedure. For example, in some western Canadian oil fields, the proportion of water in the oil-water emulsion brought to the surface can be 95% by volume (personal communication with oil field workers). That is, the volume of water handled is 19 times greater than the volume of oil produced.

Water used as injection water can be of three types formation water, sea water or fresh water. Formation water is subsurface brackish or brine water produced from a petroleum or non-petroleum producing formation. Sea water may also include water from a salty (non-potable) lake. Fresh water, containing less than 2000 ppm dissolved solids, is

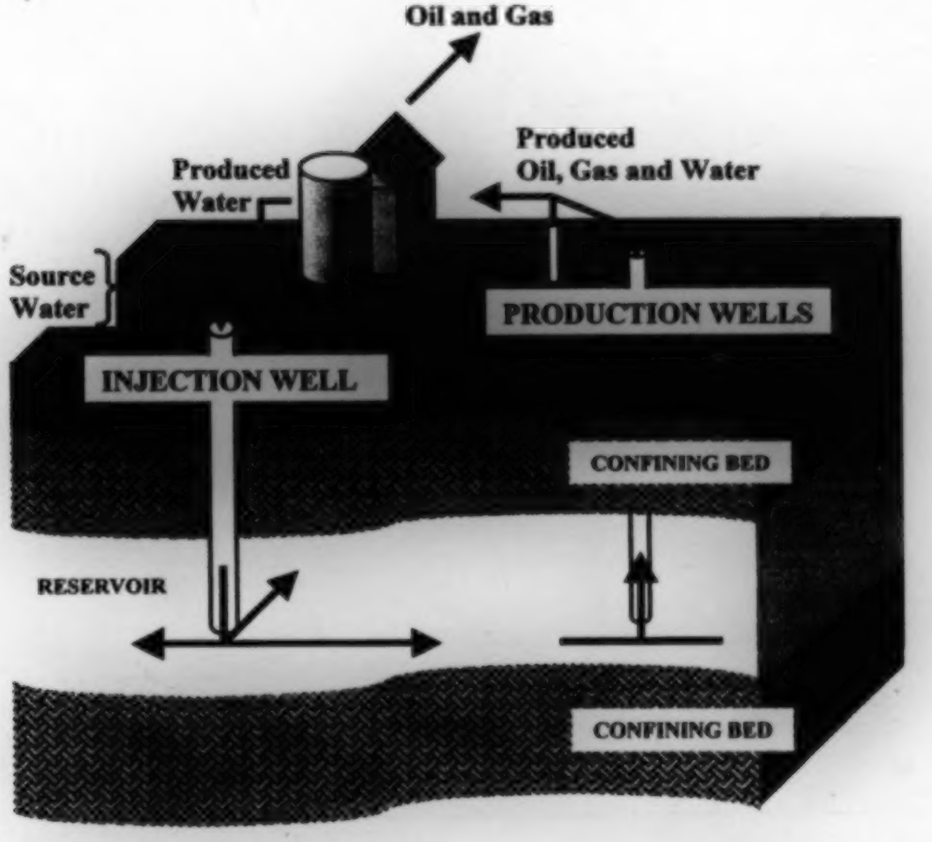

Figure 1.2 A simple waterflooding operation. Oil, gas and water are collected from the production wells, and the produced water is separated from the oil and gas. The produced water is combined with source water and injected into the oil-bearing rock to pressurize the formation and sweep the oil to the producing wells.

primarily water that can be made potable by flocculation, filtration and chlorination (Collins and Wright 1985).

Because oil field reservoir rocks are porous, they are susceptible to plugging by solids suspended in or precipitated from an injection fluid (Wright and Chilingarian 1989). This makes water quality testing necessary to determine parameters, such as amount and composition of suspended solids, clay sensitivities, presence of bacteria, compatibility of two or more waters and compatibility of the injection solution with reservoir rock. An example of incompatible waters occurs when sulfate scales, such as barium sulfate, calcium sulfate or strontium sulfate, are formed by mixing waters containing $SO_4^=$ with waters containing barium, calcium or strontium ions (Wright and Chilingarian 1989). As well, the gases O_2, H_2S and CO_2 found in injection waters and implicated in corrosion (Collins and Wright 1985, Wright and Chilingarian 1989), must be monitored. Water quality testing should be continued after the enhanced oil recovery operation has started to ensure that the system is maintained at optimum conditions (Collins and Wright 1985). Water treatment methods are outlined by Rose et al. (1989). Water should be free of bacteria that can cause corrosion (Collins and Wright 1985, Wright and Chilingarian 1989) or plugging of equipment and injection wellbores (Collins and Wright 1985). The presence of bacteria can be problematic because they reproduce rapidly over wide ranges of pH, temperature, pressure and anoxia in the reservoir. Problem bacteria, found in oil field injection waters, are SRB, iron-reducing bacteria and slime-formers (Collins and Wright 1985, Rose et al. 1989). Of special concern are the SRB.

There are several reasons why source waters used in waterflooding can increase the activities of SRB souring (Farquhar 1998). The source water, especially sea water, may contain $SO_4^=$ to serve as a terminal electron acceptor and may introduce SRB, nutrients, such as short chain fatty acids, and NH_4^+ into the reservoir. Large volumes of source water may reduce the salinity and temperature in the formation near the injection well, providing an environment that is more conducive to the growth of SRB and oil field souring.

1.4 Sulfate-reducing bacteria

Ask any person who works in the oil field or who is involved with the transport or storage of crude oil to name some bacteria and most will immediately respond "sulfate-reducing bacteria" or "SRB". These bacteria are well-known, and in the oil field environment, they are a nuisance because their metabolic activities produce H_2S that can sour reservoirs, create plugging through FeS formation and induce corrosion (Jack 1993). SRB have the unique ability to utilize $SO_4^=$ as a terminal electron acceptor. This is an anaerobic respiratory process used to generate energy for the biosynthetic reactions involved in cell growth and maintenance (Akagi 1995).

The SRB are a diverse group of prokaryotes that are found in many anaerobic environments. These bacteria have been the subject of several books (Postgate 1979, 1984, Odom and Singleton 1993, Barton 1995) and countless articles. The phylogeny of SRB has recently been reviewed (Castro et al. 2000), and based on rRNA sequences, they fall into four groups: Gram-negative mesophiles, Gram-positive endospore-formers, thermophilic bacteria and thermophilic Archaea.

1.4.1 Overview of the metabolism of SRB

The dissimilatory H_2S-producing SRB have little energy available to them. The upper limits of energy conservation from sulfate reduction are set by thermodynamics. For example, if a potent electron donor like H_2 is oxidized, the free energy change of the overall reaction, under standard conditions at neutral pH, is –38 kJ (mole H_2)$^{-1}$ (reaction 1.1). This free energy change is 6-fold lower than with O_2 as the terminal electron acceptor (reaction 1.2) (Cypionka 1995).

$$4H_2 + SO_4^{=} + 2H^+ \rightarrow H_2S + 4H_2O \qquad \Delta G^{0'} = -38\ \text{kJ}\ (\text{mol}\ H_2)^{-1} \qquad (1.1)$$

$$4H_2 + 2O_2 \rightarrow 4H_2O \qquad \Delta G^{0'} = -237\ \text{kJ}\ (\text{mol}\ H_2)^{-1} \qquad (1.2)$$

As late as the 1970's, only a few genera of SRB were recognized. These SRB were known to use only a few growth substrates, most notably lactate, pyruvate or H_2. Now it is apparent that SRB are capable of using various compounds as electron donors.

Based on their metabolic capabilities, heterotrophic SRB fall into two groups: those that cannot oxidize acetate and those that carry out complete oxidation of acetate to CO_2 (Pfennig et al. 1981). Reaction (1.3) illustrates the overall reaction of lactate-utilizing SRB that cannot oxidize acetate. One mol of acetate accumulates for each mol of lactate that is consumed.

$$2CH_3CHOHCOO^- + SO_4^= + 2H^+ \rightarrow 2CH_3COO^- + 2H_2O + 2CO_2 + H_2S$$

$$\Delta G^{o\prime} = -77 \text{ kJ (mol lactate)}^{-1} \qquad (1.3)$$

The complete oxidation of acetate is given by reaction (1.4), showing that less energy is available per mol of acetate than per mol of lactate (reaction 1.3).

$$CH_3COO^- + SO_4^= \; 3H^+ \rightarrow 2CO_2 + H_2S + 2H_2O$$

$$\Delta G^{o\prime} = -41 \text{ kJ (mol acetate)}^{-1} \qquad (1.4)$$

Increased understanding of the metabolic diversity of SRB now indicates that nearly 100 organic compounds can be used by various SRB (Barton and Tomei 1995). These substrates include fatty acids up to C_{20}; aromatic hydrocarbons, such as toluene, xylenes, ethylbenzene and naphthalene; *n*-alkanes from (C_6 to C_{20}) and simple oxidation products of hydrocarbons, such as benzoate, phenol and cresol (Hansen 1993; Widdel and Rabus 2001; Heider et al 1999). These substrates are present in native crude oils or partially degraded crude oils. Thus, if there is an ample supply of $SO_4^=$ in water contacting crude oil in an anaerobic environment, there is the potential for SRB to actively produce H_2S, using many different organic compounds (or H_2) as an energy source.

The ability to reduce $SO_4^=$ links this diverse group of bacteria. However, it is now apparent that various SRB can reduce other chemical species including Fe(III), NO_3^-, some chlorinated aromatics, sulfur oxyanions and O_2 (Barton and Tomei 1995). Molecular oxygen can be reduced by most SRB. In this case, the stoichiometry (for example, $2H_2$ consumed per O_2 reduced) indicates that O_2 can be completely reduced to water.

SRB are also capable of fermentative growth or utilization of other electron acceptors, such as sulfite, thiosulfate, elemental sulfur (McInerney et al. 1993, Cypionka 1995) and tetrathionate (McInerney et al. 1993). Many SRB are able to ferment organic substrates like pyruvate, lactate and propionate in the absence of $SO_4^=$. *Desulfotomaculum orientis* has been shown to carry out homoacetate fermentation also known as carbonate respiration. Many SRB can perform a unique fermentation of inorganic sulfur compounds which are disproportionated to $SO_4^=$ (a more oxidized compound) and sulfide (a more reduced compound). For example, thiosulfate is transformed to equal amounts of $SO_4^=$ and sulfide, and sulfite is disproportionated to 3/4 $SO_4^=$ and 1/4 sulfide (Cypionka 1995).

Some species of SRB are able to utilize NO_3^- as an electron acceptor. When NO_3^- is used as an electron acceptor, SRB produce NH_4^+ and not N_2 as an end product. Nitrite is formed as an intermediate of nitrate reduction and can be reduced by many sulfate reducers unable to reduce NO_3^-. In the presence of both $SO_4^=$ and NO_3^- some SRB will preferentially use one or the other as an electron acceptor, and some SRB will reduce both concomitantly (Cypionka 1995).

1.4.2 Activities of SRB in anaerobic environments

When microorganisms get into stagnant or closed water systems with sufficient carbon source, dissolved O_2 is quickly and completely consumed. Despite the absence of O_2, organic matter may undergo biological decomposition by microbial activities, including fermentation. The degradation reactions by which most fermentative bacteria gain energy are disproportionations of the organic matter, part converted to CO_2 and part converted to reduced products, such as fatty acids, H_2 and alcohols (Cord-Ruwisch et al. 1987). If $SO_4^=$ is abundant in these anaerobic environments, the fermentation products are used by SRB. Sulfate serves as the terminal electron acceptor, and the reducing power from the decomposed organic matter results in the formation of H_2S.

SRB grow in anaerobic muds found in fresh water or sea water environments (White 1995). They are also indigenous members of the microbial community in groundwaters, marine environments, coastal sediments, marine hydrothermal vents associated with volcanic or tectonic activity and hot springs (Azadpour et al. 1996). SRB can flourish in environments wherever decomposable organic matter gets into anaerobic, sulfate-containing waters. Here H_2S is produced and evidenced by visible blackening of the sediment when FeS forms from iron minerals (Cord-Ruwisch et al. 1987). Marine and estuarine saltmarsh sediments, saline and hypersaline lakes and ponds and oil field waters with high $SO_4^=$ content are the most permanent and significant habitats of SRB (Faugue

1995). Large amounts of $SO_4^{=}$ are required for this process, so that the consequence is the dissemination of massive quantities of H_2S (Akagi 1995), resulting from the growth of SRB.

Many SRB use simple, low molecular weight compounds and therefore depend on fermentative bacteria to cleave and ferment complex organic matter. SRB convert only about 10% of the total substrate carbon to cellular material, so that the bulk of the substrate has to be decomposed for providing energy. Thus, SRB make themselves conspicuous by the formation of their metabolic product, H_2S, rather than by formed cell mass (Cord-Ruwisch et al. 1987).

How do SRB become so closely linked to oil recovery processes? Some think that SRB are imported with surface waters or groundwaters. This hypothesis is illustrated by a gradual increase of sulfide production after the beginning of operations in oil fields (Cord-Ruwisch et al. 1987). Azadpour et al. (1996) reported that SRB were absent in 13 core samples of petroliferous formations obtained from a wide variety of geographical locations, depths and types of formations. Produced waters from six of the wells were also tested and five were positive for SRB. Acetate-utilizing SRB of the genus *Desulfobacter* were found in an oil field sea water injection system (Brink et al. 1994). In culture, they produced extensive biofilm and exhibited high levels of hydrogenase activity, which suggests a sessile habit and a role in the cathodic depolarization mechanism of microbially-influenced corrosion. Others have suggested that deep terrestrial subsurface reservoirs contain active and diverse populations of microorganisms, including SRB (McInerney et al. 1993). Thermophilic SRB isolated from oil field waters in the Norwegian sector of the North Sea were thought to be indigenous to the reservoir (Rosnes et al. 1991). See Magot et al. (2000) for discussion of microorganisms and oil reservoirs.

1.4.3 Controlling SRB in oil fields using biocides

Virtually all oil field water systems contain some bacteria (Rose et al. 1989), and biocides are widely used to kill or inhibit the activities of these microorganisms, including SRB. There are two general types of biocides oxidizing and non-oxidizing.

Typically, oxidizing biocides (such as chlorine, sodium hypochlorite, chlorine dioxide, chloroamines and bromine) are used in fresh water systems, whereas non-oxidizing biocides (including aldehydes, quaternary amines, halogenated organics, organosulfur compounds and quaternary phosphonium salts) are used in many different types of water systems (Boivin 1995).

Biocide application in large waterflooding systems presents problems, such as high cost, environmental risks (Cord-Ruwisch et al. 1987) and worker safety. The use of biocides is most successful in controlling unwanted activities in surface facilities. When used to eliminate bacteria in injection water or kill SRB in the formation, the degree of difficulty and expense increases significantly (McInerney et al. 1993). Nonetheless, application of biocides is the most common method of controlling microbial activities in the oil field. Jack and Westlake (1995) reviewed the control of SRB in the petroleum industry.

1.5 Nitrate-reducing bacteria

1.5.1 Types of NRB

There are two major groups of bacteria that could be stimulated by the presence of NO_3^- in anaerobic environments. These are chemoorganotrophs (heterotrophs) that use organic compounds as electron donors and as their carbon source for growth (Figure 1.3) and chemolithotrophs (autotrophs) that typically use reduced inorganic sulfur species as electron donors and CO_2 as their carbon source for growth (Figure 1.4). The latter group is also known as the "colorless sulfur bacteria". Figures 1.3 and 1.4 show some of the characteristics of these NRB and their end products from nitrate reduction. These figures broadly represent the types of bacteria that might be stimulated by NO_3^-, although some, such as ***Thiobacillus denitrificans*** and ***Paracoccus pantotrophus*** (Figure 1.4), do not appear to have been described as oil field bacteria. ***Pseudomonas stutzeri*** is given as an example of a heterotrophic NRB (HNRB) that might be stimulated by NO_3^- (Figure 1.3). A nitrate-respiring bacterium that has 100% similarity to ***P. stutzeri*** was isolated from an enrichment from water injectors in a North Sea oil field (Myhr et al. 2002).

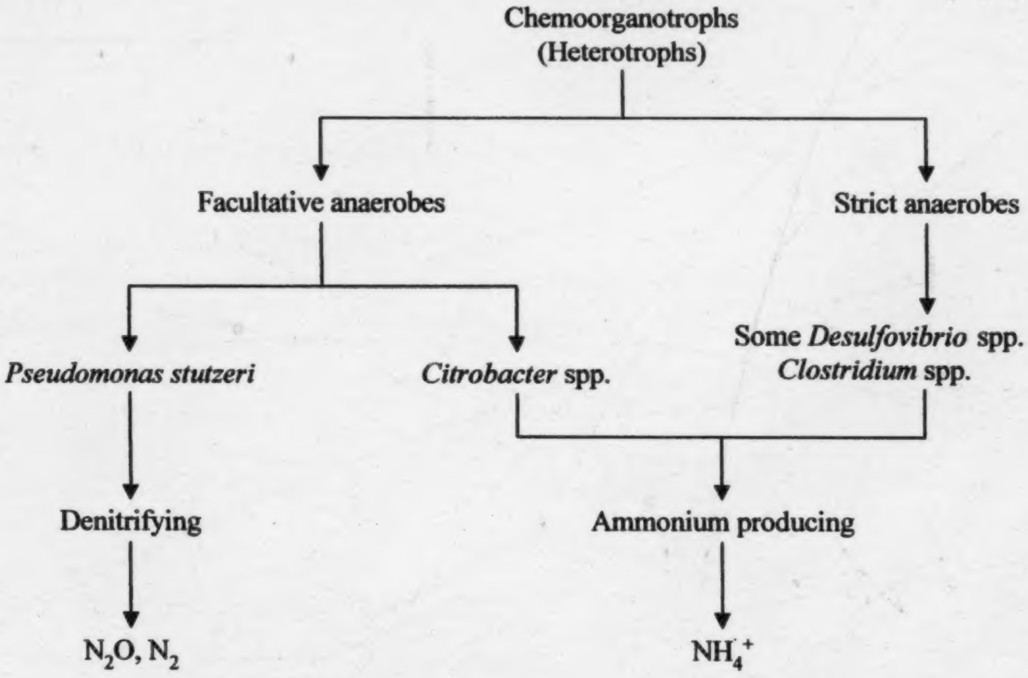

Figure 1.3 Examples of some heterotrophic bacteria that could be stimulated by the presence of NO_3^- in anaerobic environments that contain suitable organic substrates.

Among the heterotrophs in Figure 1.3 are facultative anaerobes (such as some *Pseudomonas* and *Bacillus* species) that prefer to grow using O_2 as their terminal electron acceptors but will grow using NO_3^- as their terminal electron acceptor in the absence of O_2. These are known as denitrifying bacteria, yielding N_2 as the major end product of NO_3^- respiration. There have been countless studies of denitrifying bacteria in soils and wastewater treatment, but these bacteria have been largely ignored in oil field studies. Denitrifying bacteria have been shown to degrade a variety of hydrocarbons (for review see Heider et al. 1999; Widdel and Rabus 2001), and with the abundant supply of dissolved hydrocarbons in produced waters, these heterotrophs may be stimulated by nitrate injection into a reservoir.

Another group of heterotrophic facultative anaerobes is the dissimilatory ammonium-producing NRB, such as *Citrobacter* spp. (Figure 1.3); other members of *Enterobacteriaceae* and a few other genera (Tiedje 1988). There appears to be no investigation that has specifically described ammonium production in oil field waters by this group of facultative anaerobes. However, Telang et al. (1997) mentioned an oil field isolate (designated NH15b) that was tentatively identified as a *Citrobacter* sp. or *Salmonella* sp. These would have the potential to reduce NO_3^- to NH_4^+. Recently, the strictly anaerobic ammonium-producing NRB, *Denitrovibrio acetiphilus*, was isolated from an oil reservoir model column, and this NRB was shown to produce NH_4^+ in medium that contained acetate and NO_3^- (Myhr and Torsvik 2000).

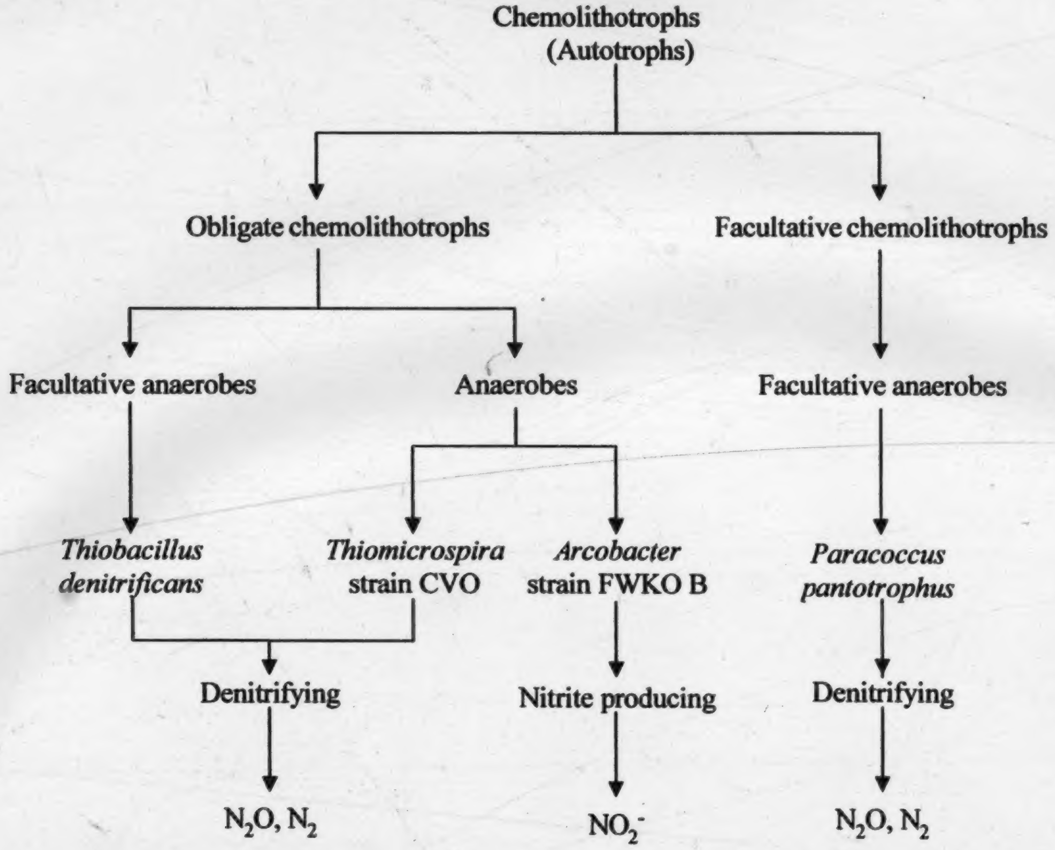

Figure 1.4 Examples of some chemolithotrophic bacteria that could be stimulated by the presence of NO_3^- in anaerobic environments. See text for details.

Some SRB (*Desulfovibrio* spp.) have also been included as heterotrophs that might be stimulated by the addition of NO_3^- (Figure 1.3) because a few of these reduce NO_3^- to NH_4^+ (McCready et al. 1983, Mitchell et al. 1986; Seitz and Cypionka 1986, Dalsgaard and Bak 1994). In the presence of NO_3^-, some SRB will preferentially use NO_3^-, and some SRB will use both concomitantly (Mitchell et al. 1986).

Thiobacillus denitrificans is listed as one of the chemolithotrophs in Figure 1.4. In general, this species does not have a tolerance for high sulfide concentrations, but Sublette and Woolsey (1989) enriched *Thiobacillus denitrificans* strain F that initially tolerated up to 1.75 mM sulfide. Later, this strain tolerated up to 2.5 mM sulfide (Sublette et al. 1994). Strain F has been used in studies to demonstrate its ability to reduce H_2S concentrations in porous rock cores (McInerney et al. 1992, 1996) and in sour produced waters (Sublette et al. 1993, 1994).

Gevertz et al. (2000) described two novel bacterial isolates that are obligate chemolithotrophs, using NO_3^- as a terminal electron acceptor and sulfide as an energy source. Both grow under anaerobic conditions. One isolate is a denitrifier that closely resembles *Thiomicrospira denitrificans* and it has been called *Thiomicrospira* strain CVO (Figure 1.4). The other isolate was called *Arcobacter* strain FWKO B and it reduces NO_3^- to NO_2^-. As well, injection of NO_3^- into an oil field might also stimulate the activity of bacteria similar to *P. pantotrophus* (Rainey et al. 1999) [formerly *Paracoccus denitrificans* (Ludwig et al. 1993) and *Thiosphaera pantotropha* strain GB17 (Robertson and Kuenen 1983)]. This bacterium was isolated from a denitrifying effluent treatment system. It is a facultative anaerobe and facultative autotroph (Figure 1.4) that uses NO_3^- as an electron acceptor. It grows chemolithotrophically with sulfide as an electron donor or heterotrophically with a variety of organic compounds [including acetate, commonly found in produced waters (Carothers and Kharaka 1978, Barth 1991)] as electron donors (Robertson and Kuenen 1983). There appears to be no research that has detected facultative chemolithotrophs in oil field waters.

The bacteria shown in Figure 1.4 all have the capability of oxidizing sulfide while reducing NO_3^-. These are referred to as nitrate-reducing, sulfide-oxidizing bacteria (NR-SOB). Greene et al. (2003) compared the sulfide tolerance of four species of NR-SOB. In their liquid medium, when sulfide was oxidized, the concentrations tolerated were less than 0.5 mM by *Thiobacillus denitrificans* strain F, up to 3 mM by *Thiomicrospira denitrificans* and *Arcobacter* sp. strain FWKO B and up to 15 mM by *Thiomicrospira* strain CVO.

Although only a few NR-SOB have been identified in oil field waters, Loka Bharathi et al. (1997) isolated over 100 strains of anaerobic colorless NR-SOB from sea water and a sulfide-rich creek. Their data showed that different isolates oxidized sulfide at different rates. For example, one isolate oxidized all of the sulfide in the medium within 9 d, whereas another isolate oxidized only 2.9% of the sulfide in the same time. Thus, it is likely that different NR-SOB in the produced water from oil fields would oxidize sulfide at different rates.

1.5.2 NRB in oil field waters

The presence of NRB in oil field waters has not been studied extensively. This group of microorganisms was not even mentioned in a review entitled "Microbiology of petroleum reservoirs" (Magot et al. 2000). Several investigations have enumerated NRB in oil field waters using most probable number (MPN) methods with different media formulations. Some of the results are summarized in Table 1.1, in chronological order. One of the first enumeration studies (Adkins et al. 1992) used molasses or sucrose as electron donors in the media to count HNRB in samples taken as near the wellheads as possible. Very low numbers ($\leq 4\ L^{-1}$) were found in these samples.

Most of the other media formulations preferentially but not exclusively cultured autotrophs. For example, the medium used by Davidova et al. (2001) (Table 1.1) contained only inorganic compounds except for yeast extract, with thiosulfate serving as the electron donor. This would preferentially grow microorganisms that are similar to *Thiobacillus denitrificans*. Other investigations listed in Table 1.1 used sulfide as the electron donor in filter-sterilized produced water from the oil field that was being studied (Gevertz et al. 1995, Telang et al. 1997). The filtered produced water undoubtedly contained some dissolved organic compounds, so it would support the growth of HNRB and chemolithotrophic NRB. The medium used by Telang et al. (1999) listed in Table 1.1 contained only inorganic compounds except for acetate, with sulfide serving as the electron donor.

Telang et al. (1999) in Table 1.1 described the isolation and characterization of two chemolithotrophic NR-SOB from an oil field in Saskatchewan, Canada. One was

Table 1.1 Detection and enumeration of NRB in oil field waters.

Ref.	Oil fields	Methods	Comments
1.1	Oklahoma, USA	MPN with molasses and sucrose as electron donor	Samples collected near wellheads. Medium would detect HNRB. MPN values were ≤4 mL^{-1}.
1.2	Saskatchewan, Canada	Single-bottle MPN using filter-sterilized oil field water supplemented with NO_3^-	Oil field water contained about 120 mg sulfide L^{-1}. Method likely selected for NR-SOB. Initial count, 10^4 mL^{-1}. Count after nitrate injection into reservoir, 10^8 mL^{-1}.
1.3	Saskatchewan, Canada	Single-bottle MPN using filter-sterilized oil field water supplemented with NO_3^-	Oil field water contained about 100 mg sulfide L^{-1}. Method likely selected for NR-SOB. Initial counts as low as 0 mL^{-1}. Counts after nitrate injection into reservoir, as high as 10^8 mL^{-1}.
1.3	Saskatchewan, Canada	Reverse Sample Genome Probing	NR-SOB strain CVO became dominant community member after nitrate injection into reservoir.
1.4	Western Canada and west Texas, USA	Single-bottle MPN using medium with sulfide, acetate and NO_3^-	Method likely selected for NR-SOB, but may have grown HNRB. Counts from 10^2 mL^{-1} to 10^6 mL^{-1} in five samples examined.
1.4	Western Canada & west Texas, USA	Reverse Sample Genome Probing	NR-SOB strains CVO and FWKO B detected in only one of five samples examined.
1.5	Oklahoma, USA and Alberta, Canada	MPN with inorganic salts, yeast extract and thiosulfate as the electron donor.	Method likely selected for thiosulfate-oxidizing NRB, but may have grown HNRB. Counts were typically <500 mL^{-1}.
1.6	Veslefrikk in the North Sea	MPN with acetate, butyrate, caproate and lactate as carbon sources.	Sampled biofilms on coupons in water injection system. Prior to nitrate injection, 10^3 NRB cm^{-2}, after 18 months of nitrate injections, a 60,000-fold increase in NRB occurred.

References (Ref.): 1.1: Adkins et al. (1992); 1.2: Gevertz et al. (1995); 1.3: Telang et al. (1997); 1.4: Telang et al. (1999); 1.5: Davidova et al. (2001); 1.6: Thorstenson et al. (2002).

designated *Thiomicrospira* strain CVO [formerly *Campylobacter* strain CVO, (Telang et al. 1997)] and the other was designated *Arcobacter* strain FWKO B. The DNA from these two isolates has been used extensively with a method known as reverse sample genome probing (RSGP), first described by Voordouw et al. (1991). Using RSGP, Telang et al. (1997) (Table 1.1), demonstrated that the abundance of strain CVO increased after the waterflooded oil field was treated with NO_3^-. This molecular technique corroborated the increase in NR-SOB numbers determined by the MPN method. The high specificity of the RSGP for NR-SOB precluded the detection of other NRB in samples from four additional oil fields from western Canada and west Texas (Telang et al. 1999), although culture methods detected NRB (Table 1.1).

NRB were detected in biofilms on coupons in the anaerobic part of the water injection system of the Veslefrikk field in the North Sea (Thorstenson et al. 2000) (Table 1.1). The medium used to enumerate these attached bacteria contained organic acids as carbon sources, providing counts of HNRB. These numbers increased dramatically after nitrate injection (Table 1.1).

Each of the oil fields mentioned in Table 1.1 contained detectable numbers of NRB at one or more sampling locations. Thus, each field had a microbial community containing NRB with the potential to be stimulated by nitrate amendment.

1.6 Controlling microbial production of sulfide with NO_3^- addition

1.6.1 Microbial mechanisms leading to the control of sulfide concentrations after NO_3^- addition

There appear to be five mechanisms by which sulfide concentrations can be controlled in the presence of NO_3^- and $SO_4^=$. The first involves the competition between HNRB and SRB for a common electron donor. For example, acetate serves as an electron donor for NRB (Beauchamp et al. 1989) and for several genera of SRB (Castro et al. 2000). Equations (1.5) and (1.6) illustrate that if acetate is available, nitrate reduction yields more energy per mol of electron donor or acceptor than does sulfate reduction (Thauer et al. 1977).

$$5CH_3COO^- + 8NO_3^- + 3H^+ \rightarrow 10HCO_3^- + 4N_2 + 4H_2O$$

$$\Delta G^{o\prime} = -495 \text{ kJ (mol } NO_3^-)^{-1} \text{ or } -792 \text{ kJ (mol acetate)}^{-1} \quad (1.5)$$

$$CH_3COO^- + SO_4^{=} \rightarrow 2HCO_3^- \; HS^-$$

$$\Delta G^{o\prime} = -47 \text{ kJ (mol acetate or } SO_4^{=})^{-1} \quad (1.6)$$

Thus, HNRB out-compete heterotrophic SRB for electron donors, thereby suppressing sulfide production. Oil field waters contain dissolved organic compounds including short-chain fatty acid anions, such as acetate, propionate and butyrate (Barth 1991, McInerney et al. 1993, Magot et al. 2000) as well as aromatic compounds, such as toluene and phenols that are substrates for heterotrophs. This mechanism would stop sulfide production but it would not remove sulfide that is present in the reservoir or produced waters.

A second mechanism results from the increased redox potential of an aqueous environment caused by the activities of denitrifying bacteria (Jenneman et al. 1986a). The production of N_2O and maybe NO raises the redox potential to above –100 mV, which is too high for the growth of SRB (Postgate 1984). Nitrate reduction in laboratory experiments causes the redox indicator, resazurin, to turn from colorless to pink (Jenneman et al. 1986a, 1986b). Resazurin is 50% oxidized at –51 mV (Jacob 1970). This alteration of the redox potential in an aqueous environment inhibits sulfide production.

A third mechanism results from the stimulation of NR-SOB in the presence of NO_3^-. Two processes come into play in this case. Some NR-SOB are denitrifiers, produce N_2O from NO_3^- and elevate the redox potential of the medium (Jenneman et al. 1986a). In addition, the NR-SOB use sulfide as their electron donor and oxidize it to elemental sulfur or $SO_4^{=}$ (Jenneman et al. 1996). Thus, these two processes combine to inhibit sulfate reduction and remove sulfide that is present in the aqueous environment. The activities of the NR-SOB have the potential to stop sulfide production and remove essentially all of the sulfide in the aqueous environment.

A fourth mechanism is nitrate reduction by SRB. Some SRB reduce NO_3^- to NH_4^+ (McCready et al. 1983, Mitchell et al. 1986, Seitz and Cypionka 1986, Dalsgaard and Bak 1994). The importance of this mechanism in controlling sulfide production is largely unexplored. Jenneman et al. (1986a) point out that when SRB reduce NO_3^-, NH_4^+ is formed rather than N_2O or N_2. The formation of N_2O would be detrimental to the SRB as discussed above.

The fifth mechanism is the production and accumulation of NO_2^- during nitrate reduction. Myhr et al. (2002) demonstrated that the activity of the dominant sulfate-reducing strain found in their laboratory experimental system was inhibited by 120 μM NO_2^-. However, some species of SRB contain nitrite reductase which reduces NO_2^- to NH_4^+ (Moura et al. 1997), thereby protecting these species from the NO_2^- produced by NRB (Greene et al. 2003).

1.6.2 Control of sulfide in wastewaters

Long before NO_3^- addition was considered for controlling sulfide in oil field waters, it was used to control odors in wastewaters and receiving surface waters. For example, in 1931, a combination of $NaNO_3$ and chlorinated lime was used to control odors from Coney Island Creek in New York (Carpenter 1932). This creek was described as "one of the vilest bodies of water in the United States" (Carpenter 1932) as a result of receiving 6,000,000 gallons (23,000,000 L) of sewage and industrial wastewater. After the first day of chemical application, there was a marked decrease in odor. During the month-long treatment, 10 tons (9 Mg) of $NaNO_3$ were applied to the creek, and the sulfide concentrations in the water decreased sharply.

Table 1.2 summarizes five studies, in which NO_3^- was used to control odors and sulfide production in wastewaters. In the first four studies (Table 1.2) the activities of native NRB were enhanced by adding NO_3^-. Two of these were large scale projects that involved NO_3^- applications to a river (Lawrance 1950) and to a sludge storage lagoon (Poduska and Anderson 1981) for odor control. The other three studies were laboratory-scale investigations using sewage sludge (Jenneman et al. 1986a), oily sludges from naval operations (Londry and Suflita 1999) and aqueous solutions of sulfide (Sublette et al. 1994). The latter report described work in which *Thiobacillus denitrificans* was initially used to oxidize sulfide, and later, *Thiobacillus denitrificans* strain F was used because of its tolerance to higher sulfide concentrations.

Table 1.2 Laboratory and field studies using NO_3^- to control sulfide production in wastewaters

Ref.	Summary
2.1	Three pulp mills discharged sulfite wastes into the Androscoggin River in Maine, USA. This resulted in H_2S production in the river and odor problems in nearby towns. In 1949, a total 641 tons (582 Mg) of $NaNO_3$ were added to the river. This controlled H_2S production and odors. Most of the NO_3^- was reduced to NH_4^+.
2.2	To control odor, waste sodium nitrate liquor (containing both NO_3^- and NO_2^-) was added to a storage lagoon that held aerobically digested waste activated sludge. Initially, the redox potential of the water was near –100 mV, but after several months of NO_3^- addition, it rose to near +300 mV. There was low odor potential when the redox was above +100 mV. Acetate concentrations decrease in the lagoon, and N_2 production from denitrification provided mixing within the sludge.
2.3	Laboratory studies were done with a 10-fold dilution of sewage sludge amended with 20 mM $SO_4^{=}$ and one of three electron donors: glucose, acetate or H_2. The addition of 59 mM NO_3^- completely inhibited sulfide production. Nitrate, NO_2^- and N_2O were detected in the inhibited samples, and the oxidation of the redox indicator, resazurin, was attributed to the presence of N_2O. The numbers of SRB decreased with prolonged incubation of the oxidized medium.
2.4	This paper reviewed bench-scale processes developed for the sulfide removal from gases and aqueous solutions by *Thiobacillus denitrificans*. When H_2S was introduced to batch anoxic or aerobic cultures of *T. denitrificans*, the H_2S was immediately metabolized. Oxidation of H_2S to $SO_4^{=}$ was accompanied by growth. *T. denitrificans* was immobilized by co-culture with floc-forming heterotrophs, and this mixture was used to treat water that was contaminated with sulfide. The sulfide-active floc was stable for 5 months of operation with no external organic carbon required to support the growth of the heterotrophs. *T. denitrificans* strain F, which tolerates higher sulfide concentrations, was also used in some studies.
2.5	Oily sludge from a settling tank at the US Navy Craney Island Fuel Depot in Virginia, USA was placed in serum bottles and amended with NO_3^-, stimulating indigenous NRB. Sulfate reduction was diminished with 50 mM NO_3^-, and sulfide accumulation was prevented with as little as 16 mM NO_3^-. Nitrite and N_2O were products of nitrate reduction. Sulfide was oxidized to sulfur or $SO_4^{=}$. The results indicated that NO_3^- would be useful for preventing sulfide formation in oily wastes produced onboard marine vessels.

References (Ref.): 2.1: Lawrance (1950); 2.2: Poduska and Anderson (1981);
2.3: Jenneman et al. (1986a); 2.4: Sublette et al. (1994);
2.5: Londry and Suflita (1999).

Each of the attempts to control odor or sulfide production listed in Table 1.2 was successful. One of the studies (Poduska and Anderson 1981) observed that nitrate amendment led to increased redox potential followed by a reduction in odor. The increased redox potential was observed in another study (Jenneman et al. 1986a), and this increased redox potential was attributed to the microbial production of N_2O. The increase in redox potential to above –100 mV would inhibit growth of SRB.

1.6.3 Laboratory studies using cores or columns

Using NO_3^- to control sulfide production in a petroleum reservoir involves adding NO_3^- to the injection water and pumping it into the oil-bearing formation. To be effective, the NO_3^- must migrate into the reservoir and be consumed by NRB. The NRB may be present in the oil field or water handling system, or NRB might be deliberately added to the oil field to stimulate nitrate reduction. Several laboratory studies have been done to assess the effectiveness of this process using cores or a column of sand. Five of these studies are summarized in Table 1.3, in chronological order.

Four of the five studies in Table 1.3 detected NRB in the cores or produced waters used in the experimental systems. In the fifth study, Myhr et al. (2002) (Table 1.3, Ref. 3.5), the investigators inoculated the column with a mixture of enrichment cultures, including NRB. Two of the studies, McInerney et al. (1992, 1996), focused on the activities of thiobacilli and none were detected in the cores or waters. Inoculating these two cores with *Thiobacillus denitrificans* strain F decreased sulfide concentrations once NO_3^- was injected into the cores (McInerney et al. 1992, 1996) (Table 1.3).

Two of the studies (Jenneman et al. 1996, Reinsel et al. 1996) (Table 1.3) relied solely on the formation water as the source of NRB. One study supplemented the medium with short-chain organic acids (Reinsel et al. 1996), whereas the other study did not supplement with organic compounds (Jenneman et al. 1996). Thus, these studies likely enriched for different nutritional types of NRB. Nonetheless, souring was inhibited in both studies. Indeed, sulfide production was controlled in each of the five studies summarized in Table 1.3.

Table 1.3 Laboratory studies using NO_3^- to control sulfide production in columns or cores

Ref.	Summary
3.1	This study investigated the efficacy of NO_3^- and the sulfide-tolerant *Thiobacillus denitrificans* strain F in controlling H_2S concentrations in cores of sandstone. Formation water from a gas storage facility in Redfield, Iowa, USA was injected into two core systems, with hydraulic retention times (HRTs) of 3.2 h and 16.7 h. With the addition of NO_3^- alone, no thiobacilli were cultured from the core system, but NO_3^- was consumed and the concentrations of sulfide in effluent decreased by about 40% in the core with the shorter HRT and 98% with the longer HRT. Thus, an indigenous microbial community capable of oxidizing sulfide while using NO_3^- as the electron acceptor was present. Inoculation with strain F reduced the effluent sulfide concentrations by about 80% in the core with the shorter HRT.
3.2	The test materials for this study included core material from the St. Peter formation at Redfield, Iowa, USA and water from the same formation, supplemented with acetate and enriched with SRB to 10^7 cells mL^{-1}. The core material did not contain large numbers of organisms capable of using NO_3^-, and no strain F-like organisms were detected. When NO_3^- and strain F were injected into the core, sulfide concentrations decreased, demonstrating the ability of strain F to control sulfide in the core.
3.3	This work examined the control of microbial souring in anaerobic upflow columns containing crushed Berea sandstone maintained at 60°C. Produced waters from the Ninian North Sea and the Kuparuk North Slope (Alaska) oil fields were used as sources of microorganisms and these gave similar results. A highly anaerobic medium that contained short-chain organic acids found in the produced waters was pumped through the columns. Nitrate injection stimulated indigenous microbes and inhibited souring at thermophilic temperatures. Initially, 3.6 mM NO_3^- was needed to inhibit souring, but later, 0.36 mM NO_3^- prevented further souring. Nitrate was reduced to NO_2^-, with no N_2O, N_2 or NH_4^+ detected.
3.4	Brine from an oil field near Coleville, Saskatchewan, Canada was tested. Tests also included brine that was filtered, supplemented with phosphate and NO_3^- and these were pumped into a porous (1288 mD) ceramic core 19.1 cm long. When 5 mM NO_3^- was shut in the column, all of the sulfide was removed in 3 d, and the numbers of NRB increased. Under various flow regimes, with sulfide-containing brine, sulfide removal was between 87 and 100%. Elemental sulfur, bacteria and $CaCO_3$ were produced, but there was no significant permeability changes across the core following all treatments.
3.5	Separate enrichments of aerobic oil-degrading bacteria, NRB, SRB and methanogens were inoculated into a 200-cm column packed with oil-soaked silica sand. The column was flooded with air-saturated synthetic sea water and operated under different influent regimes for nearly 1100 d. Injecting 0.5 mM NO_3^- led to the complete elimination of H_2S. Inhibition of the SRB was attributed to the NO_2^- produced from nitrate reduction. Three strains of HNRB were isolated from the column, and none of the HNRB used H_2S or elemental sulfur as electron donor.

References (Ref.): 3.1: McInerney et al. (1992); 3.2: McInerney et al. (1996);
3.3 Reinsel et al. (1996); 3.4: Jenneman et al. (1996); 3.5: Myhr et al. (2002).

1.6.4 *Laboratory studies using natural microbial communities in produced waters*

Produced waters from various oil fields have been used as sources of planktonic microorganisms in studies for the ability of NO_3^- to control sulfide formation in these waters. Table 1.4 summarizes four of these investigations in chronological order.

Table 1.4 Laboratory studies on controlling sulfide production in produced waters by adding NO_3^- to stimulate natural microbial communities.

Ref.	Summary
4.1 & 4.2	Anaerobic enrichments were prepared by supplementing NO_3^- and phosphate to brine samples collected from an oil field near Coleville, Saskatchewan, Canada. Within 24 to 48 h after supplementation, complete oxidation of 3 to 4 mM sulfide was observed. Elemental sulfur was formed and the stoichiometry of the reaction was $5HS^- + 2NO_3^- + 7H^+ \rightarrow 5S^0 + N_2 + 6H_2O$.
4.3	Waters from four west Texas, USA oil fields were used to determine which amendments were required to stimulate sulfide removal. In two of the samples, addition of 40 mM NO_3^- and phosphate was not sufficient to promote microbial removal of sulfide over a 28-d incubation. However, sulfide removal was observed when acetate or formate plus vitamins or yeast extract were added to these two waters that had been supplemented with NO_3^- and phosphate. These results illustrate the importance of heterotrophic activity in sulfide removal.
4.4	Two waterflooded, souring oil fields in Oklahoma, USA and Alberta, Canada were studied. SRB and NRB were found in produced waters from both oil fields. The majority of the sulfide production appeared to occur after the oil was pumped aboveground, rather than in the reservoir. Sulfide production was greatest in the water storage tanks in the Alberta field. Laboratory experiments showed that adding 5 and 10 mM NO_3^- to produced waters from the Oklahoma and Alberta oil fields, respectively, decreased the sulfide content to negligible levels and increased the numbers of NRB.

References (Ref.): 4.1: Gevertz et al. (1995); 4.2: Jenneman et al. (1996);
4.3: Wright et al. (1997); 4.4: Davidova et al. (2001).

In each study, sulfide removal was stimulated by NO_3^- addition. In two of the reports, no organic supplementation was required to stimulate sulfide removal. However, in one case (Wright et al. 1997), two of the four oil field waters did not respond to amendments with inorganic nutrients (NO_3^- and phosphate). Sulfide removal was only stimulated after the addition of acetate or formate plus vitamins or yeast extract, indicating that in some cases HNRB play an important role in the process of sulfide removal.

Laboratory studies have led to field application of NO_3^- or changes to field operations. For example, the work described by Gevertz et al. (1995) and Jenneman et al. (1996) preceded the experimental injection of NO_3^- into the Coleville field in Saskatchewan, Canada (Gevertz et al. 1995; Jenneman et al. 1997, 1999), and results from laboratory studies encouraged the implementation of nitrate injection in a North Sea oil field (Thorstenson et al. 2002). Based on laboratory investigations, Davidova et al. (2001) observed that the rate of sulfide production was higher in aboveground samples than in samples collected from wellheads. At an Alberta oil field, they observed high sulfate reduction activity in water storage tanks that had retention times of 2 to 3 d, and they calculated that 80 kg of microbially produced sulfide was injected daily into the reservoir from these storage tanks. Operators of this oil field have now eliminated the long retention time in the storage tanks, which has helped to reduce souring.

1.6.5 Laboratory studies using co-cultures of bacteria

To assess the microbial dynamics and processes that occur when NO_3^- is added to communities containing NRB and SRB, Voordouw and co-workers have done several studies in which pure cultures of bacteria were mixed and monitored (Table 1.5). Their work focused on the activities of the chemolithotrophic NR-SOB *Thiomicrospira* strain CVO and *Arcobacter* strain FWKO B. In all cases, the NR-SOB proliferated with the addition of NO_3^-, and in most cases, the NR-SOB removed sulfide from the medium and caused the cessation of sulfate reduction. However, sulfate reduction was not stopped in co-cultures in which the SRB produced nitrite reductase (Greene et al. 2003) (Table 1.5). Nitrite formed during nitrate reduction is inhibitory to SRB. However, the inhibition is only transient when SRB that produce nitrite reductase reduce NO_2^- to NH_4^+ (Moura et al. 1997). This work (Greene et al. 2003) (Table 1.5) clearly demonstrated that the activities of these NR-SOB cannot control sulfide production by all SRB, although the NR-SOB can oxidize the sulfide that is formed by the SRB.

Table 1.5 Laboratory studies using co-cultures and NO_3^- to control sulfide production.

Ref.	Summary
5.1	Mixtures of strains CVO and FWKO B were incubated in medium with different concentration of sulfide. Using RSGP, it was demonstrated that CVO dominated in co-cultures with low (1 mM) sulfide, but FWKO B dominated with high (15 mM) sulfide. CVO or FWKO B were co-cultured with *Desulfovibrio* strain Lac6. Sulfide dropped from 1 mM to 0 mM in 24 h in the presence of CVO. Over a 277-h incubation, sulfide remained between 1 and 2 mM in the presence of FWKO B.
5.2	The influence of nitrate-mediated control of sulfide production on metal corrosion was studied with strain CVO and a *Desulfovibrio* strain Lac6. The corrosion rate in cultures of the *Desulfovibrio* sp. with or without NO_3^- was 0.01 mm y^{-1}. The addition of CVO to the nitrate-containing culture increased the corrosion rate to 0.07 mm y^{-1}. The same trend was observed when CVO and NO_3^- were added to a consortium of SRB from a produced water. The increased rate of corrosion was attributed to the formation of thiosulfate and polysulfide during the oxidation of sulfide.
5.3	Strain CVO was added to cultures of *Desulfovibrio* strain Lac6 that were growing in various concentrations of NO_3^- or lactate. In pure culture, sulfate reduction by the *Desulfovibrio* sp. was unaffected by NO_3^- concentrations up to 10 mM. Sulfide concentrations decreased rapidly after the addition of CVO. This effect was due to the increase in the redox potential of the medium, as indicated by the oxidation of resazurin.
5.4	Strain CVO was grown in co-cultures with four different *Desulfovibrio* strains. Two of these did not have nitrite reductase, and their growth was stopped in the presence of CVO as it produced NO_2^- and elevated the redox potential of the medium. However, two of the strains had nitrite reductase and they reduced the NO_2^- formed by strain CVO. The SRB decreased the redox potential and continued to produce sulfide. This illustrated that the action of strain CVO cannot inhibit SRB that possess nitrite reductase.

References (Ref.): 5.1: Telang et al. (1999); 5.2: Nemati et al. (2001a);
5.3: Nemati et al. (2001b); 5.4: Greene et al. (2003).

Rates of corrosion have also been studied in co-culture experiments (Nemati et al. 2001a) (Table 1.5). The addition of strain CVO and NO_3^- to a culture of *Desulfovibrio* sp. strain Lac6 accelerated the corrosion rate to 0.07 mm y^{-1}. Lacatena et al. (2003) also measured corrosion rates, but they worked with an undefined, mixed enrichment culture in produced water. In the absence of NO_3^- in produced water, the corrosion rate was 0.46 mm y^{-1}, but with NO_3^- in the produced water, the corrosion rate dropped sharply to

0.03 mm y^{-1}. Data from nitrate injection into a North Sea oil field showed that prior to nitrate injection, the corrosion rate was 0.7 mm y^{-1}, but after 4 months of nitrate injection, the rate dropped to 0.2 mm y^{-1} (Thorstenson et al. 2002). Thus, the co-culture experiments (Nemati et al. 2001a) (Table 1.5) gave results that differed from those obtained with undefined mixed cultures (Lacatena et al. 2003) and full scale operations (Thorstenson et al. 2002).

1.6.6 Oil field observations

There have been few reports of field tests or full-scale application of nitrate injection to control sulfide. Six reports are summarized in Table 1.6 (in chronological order). One report was from an oil field in Oklahoma. The reservoir brine did not have $SO_4^=$ and less than detectable levels of sulfide and SRB prior to waterflooding. After the oil field was injected with $SO_4^=$ containing brine, sulfide and SRB were detected (McInerney et al. 1993). Three reports focused on the extensive studies done on the Coleville oil field in Saskatchewan, Canada during two experimental injections (Gevertz et al. 1995; Jenneman et al. 1997, 1999; Telang et al. 1997). The microbial community in the Coleville oil field was extensively characterized using the RSGP method, and the produced water was the source of the well-studied NR-SOB, *Thiomicrospira* strain CVO and *Arcobacter* strain FWKO B. The Coleville injected brine contained the ions Cl^-, HCO_3^-, $SO_4^=$, Na^+, Mg^{2+} and Ca^{2+}. Results from NO_3^- addition to two oil fields in the North Sea have also been reported (Larsen 2002, Thorstenson et al. 2002) (Table 1.6). The oil fields were injected with seawater. The NO_3^- addition results include an 8-month study (Larsen 2002) and a long-term application, with data reported after 32 months of operation (Thorstenson et al. 2002). Numbers of planktonic NRB were monitored in the first five studies listed in Table 1.6, and numbers of sessile NRB were reported in the last study given in Table 1.6.

Three common observations were evident from the field studies summarized in Table 1.6. First, NRB were present in each of the oil field waters studied. Thus, no intentional inoculation of NRB was required to stimulate the beneficial activities of these bacteria. Second, nitrate injection stimulated the NRB. In reports in which NRB were

Table 1.6 Field studies and operations using NO_3^- to control sulfide production.

Ref.	Summary
6.1	NH_4NO_3 (45 T) was injected into a souring oil field at the Southeast Vassar Verta Sand Unit in Oklahoma, USA. At the time of injection, no NO_3^- was detected in three adjacent production wells. Forty-five days after injection, NO_3^- was detected at these wells, and the sulfide concentrations were reduced by 40 to 60%.
6.2	In 1994, a solution of NH_4NO_3 and NaH_2PO_4 was injected into three wells in the Coleville field in Saskatchewan, Canada. Prior to treatment, the produced waters from these wells contained between 52 and 160 mg sulfide L^{-1}. After injection, there were shut-in periods of between 24 and 70 h before pumping resumed. The sulfide concentrations dropped by as much as 98% of the initial concentrations, with ranges between 40 and 60% being sustained for several hours. The numbers of NRB increased by 100- to 10,000-fold.
6.3 & 6.4	In 1996, a solution of NH_4NO_3 and NaH_2PO_4 was injected into two injection wells in the Coleville field for 50 d. Two producer wells were monitored for 90 d after the injection began. After 10 d, the sulfide in the producers decreased by as much as 50 to 60% of the initial concentrations of 60 and 40 mg L^{-1}. The cumulative sulfide removal from the two producers were estimated to be 50 and 70 kg over the 90-d test period. The numbers of NRB increased at least 1000-fold during the time of nitrate injection.
6.5	Samples were taken from the Coleville field in 1996. These were taken 8 d before and 20, 55 and 82 d after the injection of a solution of NH_4NO_3 and NaH_2PO_4 began. RSGP analyses, using 47 DNA standards, showed that strain CVO became the dominant community member immediately after injection. The abundance of CVO decreased within 30 d after completion of nitrate injection.
6.6	Studies were done in the Skjold oil field in the North Sea in 2000. Three injection strategies were used. In each case, the highest NO_3^- concentrations were used at the beginning of the treatment, then the concentration was decreased. First, NO_3^- (4.5 to 1.7 mM) was injected into one well for 1 month; second, NO_3^- (3.8 to 1.8 mM) was injected into this well plus another well for 2 months; third, NO_3^- (4.4 mM to a mean of 2.8 mM) was injected into all of the other wells for 3 months. Only one of the monitored production wells showed marked reduction in H_2S. This well was in the highly fractured zone of the reservoir and NO_3^- reached it within 24 h of the start of injection. The amount of H_2S in the produced gas dropped from 240 ppm to between 30 to 60 ppm. After NO_3^- addition, the numbers of mesophilic NRB and NR-SOB increased about 10,000- and 1000-fold, respectively.
6.7	Data were presented after 32 months of adding NO_3^- to water injected from the Veslefrikk platform in the North Sea. Glutaraldehyde injection was stopped in January 1999 and replaced by continuous nitrate injection (0.25 mM). Microbial counts in biofilms were monitored, and corrosion was measured by weight loss from C-steel biocoupons. After 32 months, the numbers of SRB decreased 20,000-fold and after 18 months, the number of NRB increased 60,000-fold. Most of the NRB were heterotrophic facultative anaerobes. Sulfate-reducing activity (measured using $^{35}S\text{-}SO_4^{=}$) decrease 50-fold. Prior to NO_3^- treatment, the corrosion rate was 0.7 mm y^{-1}. This fell to 0.02 mm y^{-1} after 4 months of nitrate injection.

References (Ref.): 6.1: McInerney et al. (1993); 6.2: Gevertz et al. (1995);
6.3: Jenneman et al. (1997); 6.4: Jenneman et al. (1999);
6.5: Telang et al. (1997); 6.6: Larson (2002); 6.7: Thorstenson et al. (2002).

enumerated, their numbers increased 100- to 60,000-fold during the monitoring times. Third, nitrate injection controlled sulfide production. Each of these observations was completely predicable from laboratory studies summarized in Tables 1.3, 1.4 and 1.5.

1.6.7 U.S. Patents

The ability to stop sulfide production in oil fields or to remove sulfide from sour waters and petroleum are essential in petroleum recovery and processing. The inhibition of sulfate reduction decreases corrosion and other problems associated with SRB and provides huge cost saving to the oil field operators. Therefore, it is not surprising that several patents have been issued for the use of NO_3^- or NRB for sulfide removal or control. Table 1.7 lists some of the U.S. patents dealing with these processes.

Patent no. 4,879,240 uses a mutant strain of *Thiobacillus denitrificans* that is tolerant to elevated concentrations of sulfide and glutaraldehyde (presumably strain F) to control sulfide in environments, such as oil field injection waters, reservoirs and waste treatment of materials that contain SRB. A sulfide-tolerant strain of *Thiobacillus denitrificans* is the microbial component of patent no. 4,880,542 used to remove H_2S from sour waters originating from petroleum production, anaerobic sewage digestion or other industries. These chemolithotrophic bacteria are co-immobilized with $CaCO_3$ in alginate beads and placed in a column through which the wastewater is pumped. Nitrate or O_2 can serve as the terminal electron acceptor.

The activities of heterotrophic denitrifying bacteria are stimulated by supplementing oil field waters (or other sulfide-containing waters) with NO_3^- and an organic compound, such as acetate (patent nos. 5,405,531 and 5,750,392) (Table 1.7). This allows the NRB to out-compete the SRB for organic substrates. In addition, these patents include the addition of molybdate to further inhibit SRB.

The use of the chemolithotrophic NR-SOB *Thiomicrospira* (formerly *Campylobacter* sp.) strain CVO and *Arcobacter* strain FWKO B for the removal of sulfide from oil field brines is covered by patent nos. 5,686,293 and 5,789,236 (Table 1.7). The uses include aboveground treatment of sour waters or injection of these NR-

SOB into subterranean formations. The waters are supplemented with NO_3^- and phosphate.

Table 1.7 Examples of United States patents for the control of sulfide through the application of NRB.

Patent no.	Inventors and year	Title
4,879,240	Sublette et al. 1989	Microbial control of hydrogen sulfide production by sulfate reducing bacteria
4,880,542	Sublette 1989	Biofilter for the treatment of sour water
5,405,531	Hitzman et al. 1995	Method for reducing the amount of and preventing the formation of hydrogen sulfide in an aqueous system
5,686,293	Jenneman and Gevertz 1997	Sulfide-oxidizing bacteria
5,750,392	Hitzman et al. 1998	Composition for reducing the amount of and preventing the formation of hydrogen sulfide in an aqueous system, particularly in an aqueous system for oil field applications
5,789,236	Jenneman 1998	Process of using sulfide-oxidizing bacteria

1.6.8 Economics and advantages of using NO_3^- to control sulfide production

Based on the trial injections at the Coleville oil field in Saskatchewan, Canada, Jenneman et al. (1997) did a cost analysis for sulfide removal using different chemicals. They injected NH_4NO_3 (cost US\$0.31 kg^{-1}) and monosodium phosphate (cost US\$2.57 kg^{-1}) to stimulate NRB in the reservoir. The combined cost of these chemicals was determined to be between US\$0.76 and \$1.19 kg^{-1} H_2S removed. They compared this cost to reported costs for sulfide removal from wastewaters using hydrogen peroxide or sodium hypochlorite. With hydrogen peroxide, the estimated cost was between US\$4.40

and \$17.60 kg^{-1} H_2S removed, and with sodium hypochlorite the estimated cost was between US\$3.96 and \$13.20 kg^{-1} H_2S removed. With data from one well, Jenneman et al. (1997, presented at a conference in Texas, USA) estimated the cost of using NH_4NO_3 and monosodium phosphate to be \$0.018 $barrel^{-1}$ or \$1.80 (100 barrels) $^{-1}$ of produced water treated.

Herbert (2003) compared the costs of using NO_3^- with those of using the biocides glutaraldehyde or tetrakishydroxymethylphosphonium sulfate (THPS) for offshore oil fields. The costs did not include the cost of transporting the chemicals. The estimated prices per liter of the chemicals were US\$0.25 for NO_3^- (as a 40% solution of $CaNO_3$), \$2.50 for glutaraldehyde (as a 50% solution) and \$4.00 for THPS (as a 50% solution). Although the cost of NO_3^- was lower, the solution was continuously injected at a dose of 60 mg L^{-1}. In contrast, the two biocides were injected for 1 h, twice per week at a dose of 500 mg L^{-1}. Based on treating 200,000 barrels of produced water per d, the yearly costs for chemicals were US\$575,000 for NO_3^-, \$345,000 for glutaraldehyde and \$500,000 for THPS. Per 100 barrel of water treated, these costs become US\$0.79 and \$0.47 and \$0.68, respectively.

From these two cost analyses, the use of NO_3^- for sulfide control is competitive with other chemicals. The cost of treating 100 barrels of water calculated from the data given by Jenneman et al. (1997) is higher than that reported by Herbert (2003) because Jenneman et al. (1997) also injected monosodium phosphate, which is 8 times as expensive as the NH_4NO_3. Herbert (2003) used only calcium nitrate. The need to add a phosphate source to stimulate NRB would have to be evaluated for each oil field.

Besides the cost, other factors must be considered when choosing chemicals for controlling sulfide in produced waters. Most notably, worker safety and potential environmental impact of spilled chemical must be considered. Nitrate salts are far less toxic than the biocides commonly used in oil fields and therefore, their use presents few safety issues for oil field workers. Spilled biocides have negative effects on the environment. In contrast, NO_3^- is widely used as an agricultural fertilizer, so spills on land present no major problem. Nitrate is listed as a substance that poses little or no risk to the marine environment (Thorstenson et al. 2002). However, caution must be used to avoid contamination of fresh surface waters or potable groundwaters with NO_3^- (or any biocide).

1.7 Concluding remarks

The use of NO_3^- to control microbially-produced sulfide in oil fields is a proven biotechnology that is grossly under-used by the petroleum industry. Its effectiveness has been demonstrated in many laboratory investigations and in some field studies. The microbiology is adequately well-understood, although it is not clear whether heterotrophic or chemolithotrophic NRB play the more important role. This may vary from oil field to oil field. Nonetheless, from the results in the literature, nitrate amendment (and in some cases phosphate or organic acid amendment) stimulates NRB in the oil field waters, and there appears to be little need to add an inoculum of NRB.

Nitrate has replaced biocides in some of the oil fields in the North Sea, and the results have been very positive. Besides controlling sulfide levels, there is also preliminary evidence that corrosion rates are reduced (Thorstenson et al. 2002). In addition, there are plans to use NO_3^- in the Gulf of Mexico when sea water injection begins in the near future (Stephen Maxwell, Commercial Microbiology Inc., personal communication). In contrast, there is little or no use of NO_3^- in land-based souring oil fields in North America. It is now very clear that land-based oil field operators should seriously consider using this proven biotechnology to control and possibly eliminate microbially-induced souring and the problems associated with H_2S formation.

1.8 Research objectives

Many of the studies described above have shown that NO_3^- addition to sulfide-containing oil field produced waters can stimulate NRB that are capable of removing sulfide. The studies also show that in the presence of NO_3^-, sulfide concentrations are decreased and SRB are inhibited. The work to this point has focused on groups of bacteria like NRB and SRB. Where NRB have been studied, very little work has been done to determine the types of NRB that are involved in sulfide removal. The emphasis has been on the chemolithotrophic NRB, like *Thiomicrospira* strain CVO.

The primary objective for this research project was to investigate the use of NO_3^- for the control of sulfide production and removal in oil field waters. In order to fulfill this objective, four goals were set. The first goal was to find and develop methods to enumerate HNRB and chemolithotrophic NR-SOB using MPN procedures and to enumerate planktonic bacteria in oil field waters. The second goal was to monitor chemical and bacterial changes in laboratory microcosms with sulfide-containing oil field waters after NO_3^- addition. The third goal was to investigate ways to enumerate and monitor planktonic dissimilatory ammonium-producing, nitrate-reducing bacteria (DAP-NRB) in oil field waters. The final goal was to isolate oil field HNRB and NR-SOB and monitor them in sulfide-containing laboratory microcosms to determine what roles they may have during sulfide removal.

1.9 Thesis overview

The thesis is organized to give a progressive account of the work done to investigate NRB in oil field waters before and after nitrate amendment to remove sulfide. The chapters are arranged as follows. Chapter 2 details the development of a second derivative UV absorbance method that was used to show NO_3^- loss in media for MPN procedures. The second derivative UV absorbance method was used to enumerate thiosulfate-oxidizing NRB and HNRB. Chapter 3 details the methods used to enumerate planktonic bacteria in source and produced oil field waters. The enumerations were for aerobic oil field bacteria, using plate counts; and oil field HNRB, chemolithotrophic NRB and SRB, using MPN procedures. The bacteria were from five mesophilic western Canadian oil fields. The methods for collection, media preparation, enumeration and statistical comparisons are described. Chapter 4 details the methods and results for chemical and microbiological changes in laboratory microcosms after NO_3^- addition to produced waters from three western Canadian oil field locations. Chapter 5 evaluates a MPN enumeration method for DAP-NRB that was used to enumerate DAP-NRB in oil field waters before and after NO_3^- addition. Chapter 6 describes methods and results for attempts to isolate HNRB and NR-SOB from produced oil field waters. HNRB isolates and NR-SOB enrichment cultures were combined in microcosm studies with SRB (*Desulfococcus multivorans)* in order to determine roles that each NRB may play during sulfide removal. Chapter 7 gives an overall discussion of the research project described in this thesis and some suggestions for further research. The Appendix describes an experiment for observing abiotic removal of sulfide from mineral medium containing NO_2^- and N_2O.

1.10 Literature cited

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2. Second derivative UV absorbance analysis to monitor nitrate reduction by bacteria in most probable number determinations[*]

2.1 Introduction

Nitrate can be used as a terminal electron acceptor by a wide variety of bacteria called nitrate-reducing bacteria (NRB). These include chemoorganotrophs (heterotrophs) that use organic compounds as electron donors (Zumft 1992) and chemolithotrophs that use inorganic compounds, such as sulfide [*Thiobacillus denitrificans*, (Kuenen 1989), *Thiomicrospira* sp. strain CVO, (Gevertz et al. 2000)], thiosulfate [*T. denitrificans*, (Sublette et al. 1994)] or ferrous iron (Straub and Buchholz-Cleven 1998) as electron donors. Products of nitrate reduction include NO_2^-, NO, N_2O, N_2 and NH_4^+. When N_2 is produced, the process is called denitrification (Mahne and Tiedje 1995).

Because of the high redox potential of NO_3^-, NRB out-compete other microorganisms in anaerobic environments that contain NO_3^-. For example, addition of NO_3^- to fresh water sediment (Scholten and Stams 1995) or anoxic rice field soil (Chidthaisong and Conrad 2000) stopped methanogenesis. Poduska and Anderson (1981) demonstrated that NO_3^- addition to a sludge holding lagoon eliminated H_2S odor because NRB out-competed sulfate-reducing bacteria (SRB). Similarly, Jenneman et al. (1986) demonstrated that NO_3^- inhibited biogenic sulfide production in dilute sewage sludge, pond sediment and oil field brines. Recent studies have shown that NO_3^- can stimulate indigenous NRB for the control of sulfide in oil field waters (Jenneman et al. 1999).

To evaluate the potential for controlling sulfide production by adding NO_3^- to anaerobic environments, bacterial enumerations for NRB are useful to predict which types of bacteria will become active in the presence of NO_3^-. The most probable number (MPN) procedure is often used to enumerate NRB in many different environments

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(Davidova et al. 2001, Kniemeyer et al. 1999, Bekins et al. 1999, Ludvigsen et al. 1999, Adkins et al. 1992, Francis et al. 1989). The MPN method for NRB requires medium containing NO_3^- along with suitable electron donors. To complete the MPN procedure and verify the presence of NRB in MPN cultures, a NO_3^- analysis is needed to demonstrate the loss of NO_3^-. Most NO_3^- analysis techniques are too costly and time consuming to be applied to the large number of culture tubes used in a MPN procedure.

Waterflooding is a common practice used to enhance oil recovery from reservoirs. However, this process often gives rise to an increase in microbially produced H_2S which causes the oil to become "sour", thereby decreasing the value of the oil. This study was used to look at the potential to control biogenic sulfide production in western Canadian oil fields by adding NO_3^- as a competitive terminal electron acceptor and surveying the numbers of NRB in oil field produced waters, using MPN methods. Many of these waters contained high Cl^- concentrations, and the MPN media was prepared containing the same Cl^- concentration present in each oil field water. The media used for enumerating NRB in oil field waters included nutrient broth with NO_3^- to cultivate heterotrophic NRB (HNRB) and thiosulfate and NO_3^- to cultivate thiosulfate-oxidizing NRB. In both cases, a quick and reliable screening method for NO_3^- loss was needed.

The method described by Tiedje (1982) for cultivating HNRB, using the diphenylamine spot test to determine NO_3^- loss (Morgan 1930), did not always give reliable results for oil field waters. The presence of organic compounds in the medium prevented the use of ion chromatography (IC) to determine NO_3^- concentrations because organics cause fouling of the separator column (Clesceri et al. 1998). The thiosulfate-oxidizing NRB medium could be analyzed using IC. However, the presence of $S_2O_3^{2-}$ required 30-min analysis time for each MPN tube sample.

In addition, the high Cl^- concentrations often found in oil field waters interfere with methods of NO_3^- analysis, such as NO_3^- electrode methods (Clesceri et al. 1998, Mitraskas and Alexiades 1990) and gas chromatography after the conversion of nitrate to nitromesitylene (Dunphy et al. 1990). Other tests for NO_3^- that are described in Clesceri et al. (1998) indicate that organic matter interferes in IC and can influence the overall performance of capillary ion electrophoresis with indirect ultraviolet (UV) detection. Organic molecules and NO_2^- interfere with the UV spectrophotometric screening method, and the sulfide can interfere in the hydrazine reduction method. Davies et al. (1999) developed a rapid capillary electrophoresis method for NO_3^- and NO_2^- which requires removal of protein and Cl^- before testing.

The second derivative UV absorbance method used by Crumpton et al. (1992) for analyzing NO_3^- in fresh-waters was explored as a simple, quick and reliable method for measuring NO_3^- depletion in the MPN culture medium. In a complex matrix, such as culture medium, the individual components are often indistinct in UV absorbance spectra because of the large widths of component bands relative to the separations between adjacent bands. In some cases, the remedy is to calculate and plot first, second and possibly higher derivatives of the absorbance spectrum with respect to wavelength. The plots are called derivative spectra and the technique is called derivative absorbance. With this method, the first derivative defines the rates of change in the spectrum with respect to wavelength so that any broad peaks related to components in the medium are diminished and constants are removed. The second derivative then defines the rates of change in the first derivative absorbance spectrum with respect to wavelength. At this point, any sharp peaks in the spectrum will be emphasised (Cahill 1979). Using the principle for second derivative UV absorbance, a screening method was developed for the determination of NO_3^- loss in culture media for thiosulfate-oxidizing NRB and HNRB.

2.2 Materials and Methods

2.2.1 Samples for NRB enumeration

A total of five oil fields in Alberta and Saskatchewan, Canada were sampled. Source and produced waters were aseptically and anaerobically collected from September, 2000 to July, 2001. The Cl^- concentrations ranged up to 2400 mM. A domestic wastewater sample was obtained from a denitrification unit at the sewage treatment plant in Edmonton, and a water sample was collected from the North Saskatchewan River in Edmonton. These contained 1 mM and 0.1 mM Cl^-, respectively.

The NRB in the waters were enumerated using different types of media in 3-tube MPN methods. The media were prepared with Cl^- concentrations to match those of the samples. The cultures were incubated at room temperature (approximately 21°C) for 30 d in the dark. After the 30-d incubation, the media used to enumerate HNRB and chemolithotrophic NRB were tested for NO_3^- loss using second derivative UV absorbance.

The medium used for thiosulfate-oxidizing NRB was adapted from the ATCC 295 S8 (ATCC 2000) medium for Thiobacilli by reducing the amount of thiosulfate to 5 g L^{-1} and adding a trace metal solution. This medium is designated S8 medium in this work. The medium (pH 6.8 to 7.0) contained, per liter: 1.2 g Na_2HPO_4, 1.8 g KH_2PO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.1 g $(NH_4)_2SO_4$, 0.03 g $CaCl_2$, 0.02 g $FeCl_3$, 0.02 g $MnSO_4$, 5.0 g $Na_2S_2O_3 \cdot 5H_2O$, 0.5 g $NaHCO_3$, 5.0 g KNO_3, 10 mL trace metal solution; 10 mL 0.01% resazurin; 1 L boiled, distilled, deionized H_2O. The trace metal solution was made in 10.5 mM nitrilotriacetic acid (adjusted to pH 6.0 with KOH) and contained per liter: 1.0 g $MnSO_4 \cdot 2H_2O$, 0.8 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.2 g $CoCl_2 \cdot 6H_2O$, 0.2 g $ZnSO_4 \cdot 7H_2O$, 0.02 g $CuCl_2 \cdot 2H_2O$, 0.02 g $NiCl_2 \cdot 6H_2O$, 0.02 g $Na_2MoO_4 \cdot 2H_2O$, 0.02 g Na_2SeO_4, 0.02 g Na_2WO_4. This medium was sparged with O_2-free 10% CO_2, balance N_2 and dispensed into 16 x 125 mm Hungate type anaerobic culture tubes (Bellco Glass Inc., Vineland, NJ) that were flushed with the same gas. After autoclaving at 121°C for 20 min, 0.1 mL of sterile, anaerobic amorphous ferrous sulfide suspension was added as the reducing agent (Brock and O'Dea 1977). One milliliter of an appropriate dilution of a water sample in anaerobic phosphate buffer (10 mM, pH 7.2) was added to each tube giving a final volume of 10 mL of liquid in each tube. Inoculating this medium with *T. denitrificans* ATCC 23642 was used as the positive control for growth and NO_3^- consumption. In addition, sterile medium controls were made by adding 1 mL anaerobic 10 mM phosphate buffer to the S8 medium.

The medium used for HNRB was adapted from the method developed by Tiedje (1982), contained one-half (4 g L^{-1}) of the nutrient broth (BDH, Darmstadt, Germany) and 5.0 mM KNO_3. Nutrient broth-nitrate medium was tested for growth and N_2O production using full-strength (8 g L^{-1}) and one-half strength (4 g L^{-1}) nutrient broth for 16 water samples. There was no difference in the MPN values ($P<0.05$) for the two formulations comparing the 16 water samples, so the one-half strength formulation was used. The one-half strength formulation or HNRB medium was better suited for water samples and for the second derivative UV absorbance method because it contained a lower concentration of organic components. The higher concentration of organic components made the NO_3^- analysis by second derivative UV absorbance more difficult.

The HNRB medium (9 mL) was dispensed into Hungate type anaerobic culture tubes. After autoclaving at 121°C for 20 min, 1 mL of an appropriate dilution of sample water made in anaerobic phosphate buffer (10 mM, pH 7.2) was added, giving 10 mL as the final liquid volume for each tube. Sterile medium controls were prepared by adding 1 mL anaerobic 10 mM phosphate buffer to the medium. To block denitrification at N_2O (Tiedje 1982), acetylene (25% v v^{-1}) was added to the headspace of each culture tube. The headspace gas of the HNRB cultures which consumed NO_3^- were analyzed for N_2O by gas chromatography (Fedorak et al. 2002). Nitrous oxide (Praxair, Mississauga, ON, Canada) standards from 0.05 to 4% (v v^{-1}) were analyzed with each set of MPN tubes. The 4% standard was equivalent to 100% conversion of NO_3^--N to N_2O. Any MPN culture tube that contained >0.05% N_2O was considered positive.

2.2.2 *Samples for second derivative UV absorbance*

After the 30-d incubation period, the MPN culture tubes were examined for turbidity. In each case, tubes tested for NO_3^- loss included triplicate tubes for a range of dilutions showing growth in all three tubes to a dilution showing no growth in all three tubes. The NO_3^- analysis results from all these tubes were compared to results from sterile medium control tubes which gave the same NO_3^- concentration that was in the medium immediately after inoculation with the diluted water sample.

Samples from the MPN tubes were diluted before analysis by the second derivative UV method. The dilutions brought the NO_3^- concentrations into the working range of the calibration curves and reduced the chances of interference from other components in the medium. For S8 medium, cultures to be tested for NO_3^- loss were diluted 1/1000 in distilled, deionized H_2O if the Cl^- concentration was >200 mM and 1/800 if the Cl^- concentration was <200 mM. For HNRB medium, cultures to be tested for NO_3^- loss were diluted 1/300 in distilled, deionized H_2O. Initially, second derivative analyses were done comparing sample dilutions prepared before and after filtering with

0.2 μm Millex-GS Millipore filters (Bedford, MA). There was no difference between these results, so subsequent analyses used unfiltered sample dilutions.

2.2.3 Formula, instrument and wavelength for second derivative UV absorbance analysis

Equation 2.1 was used to calculate second derivative absorbance values (Cahill 1979), where, λ is wavelength in nm, and A is the absorbance measured at a given wavelength.

$$\frac{d^2A}{d\lambda^2} = \frac{A(\lambda'+\Delta\lambda) - 2A(\lambda') + A(\lambda'-\Delta\lambda)}{(\Delta\lambda)^2} \qquad (2.1)$$

For a sequence of co-ordinate pairs of λ and A, where the λ values are in steps of, for example, 1 nm, equation (2.1) was used to calculate $\frac{d^2A}{d\lambda^2}$, the second derivative absorbance, at λ', a particular wavelength. Three values of A were involved in the calculation: $A(\lambda')$, absorbance measured at λ'; $A(\lambda'+\Delta\lambda)$, measured at a wavelength which was a specified interval, $\Delta\lambda$, above λ'; and $A(\lambda'-\Delta\lambda)$, at $\Delta\lambda$ below λ'. The $\Delta\lambda$ was chosen to span several consecutive wavelengths to minimize the effect of narrow-band noise, which could otherwise be amplified by the second derivative process.

A Pharmacia Ultrospec 3000 UV, VIS spectrophotometer with a wavelength range of 190 to 1100 nm and instrument bandwidth (IBW) of 3 nm was used. The spectral bandwidth (SBW) for 69 μM NO_3^- with a A_{max}= 1.08 was 31 nm. The IBW/SBW was 0.097 which is less than 0.1 and acceptable for the Beer-Lambert law to be obeyed (Denney and Sinclair 1987). The spectrophotometer was interfaced with Swift Applications software for UV, Visible Spectrophotometers from Pharmacia Biotech, Cambridge, England 1996. Absorbance spectra from 190 nm to 250 nm were obtained for each standard NO_3^- solution and each diluted culture using quartz cuvettes with 10-mm light paths. The data were processed in Microsoft Excel using the second derivative formula, equation (2.1). All spectra for standards and cultures were obtained using distilled, deionized H_2O in the reference cell.

Second derivative UV absorbance spectra were calculated for NO_3^- and NO_2^- to determine the best wavelength to be used for NO_3^- analysis. The absorbance spectra for NO_3^- were from 215 nm to 240 nm. This range of wavelengths was used because it gave the peak of the second derivative absorbance spectra for NO_3^- and showed the wavelengths where NO_2^- could interfere in the analysis of NO_3^- (Crumpton et al. 1992).

For both S8 and HNRB media, absorbance spectra were obtained from NO_3^- standards, and calibration curves were constructed relating NO_3^- concentration to the second derivative UV absorbance. The second derivative was calculated using equation (2.1) at a wavelength of λ' 226 nm. For both media, when absorbance spectra were obtained from samples, an Excel macro was used to calculate the second derivative UV absorbance using equation (2.1). The calculations were performed on dilutions prepared from MPN tubes that had been incubating for 30 d. Comparisons were made of the second derivative UV absorbance of the growth and no-growth MPN tubes to the sterile medium control tubes. When a decrease of >20% of the NO_3^- concentration the culture medium was observed, the MPN tube was considered positive for growth of NRB. The rationale for choosing this threshold is provided in the Discussion section.

To verify the second derivative results, IC was done on the S8 medium using the Dionex IC DX600 system with an autosampler and conductivity detector. Ions were separated on an IonPac AS9-HC (240 x 4 mm) analytical column after a AG9-HC guard column (50 x 4 mm), using an eluent mixture of 9.0 mM Na_2CO_3, at a flow rate of 1.2 mL min^{-1}. PeakNet software (version 6.1) was used to integrate and calculate ion concentrations based on the peak areas of external standards. Sulfate and Cl^- concentrations were also determined by this IC method. In some cases, NO_2^- concentrations were also determined by IC. However, high Cl^- concentrations interfered with this analysis, so the colorimetric method using sulfanilamide and *N*-(1-naphthyl)-ethylenediamine dihydrochloride (Clesceri et al. 1998) was also used for NO_2^- measurements.

2.2.4 Preparation of calibration curves from second derivative UV absorbance analysis

To monitor NO_3^- concentrations in cultures grown in S8 medium, a NO_3^- calibration curve was prepared using KNO_3. Standards were diluted in distilled, deionized H_2O with concentrations of NO_3^- from 1.6 to 150 µM. Second derivative UV absorbance calculations were made using equation (2.1), and points on the calibration curve were taken from readings at 226 nm.

Because of the organic constituents in the HNRB medium, NO_3^- concentrations determined with the calibration curve prepared for analyzing HNRB medium were always higher than expected. Thus, the NO_3^- calibration curve for the HNRB medium was prepared by diluting the NO_3^- standards with NO_3^--free HNRB medium. The medium was diluted 1/300 in distilled, deionized H_2O. Appropriate amounts of KNO_3 were dissolved in this diluent to make standards with NO_3^- concentrations from 1.6 to 19 µM. Second derivative UV absorbance calculations were made using equation (2.1), and points on the calibration curve were taken from readings at 226 nm.

2.2.5 Testing for interfering substances for second derivative UV analysis

To determine whether any of the components in the defined S8 medium interfere in the second derivative UV absorbance NO_3^- analysis, uninoculated S8 medium was tested with and without NO_3^-. The medium was diluted 1/300 in distilled, deionized H_2O prior to UV analysis. To determine if Cl^- interfered with the second derivative UV absorbance analysis, tubes of S8 medium with Cl^- concentrations ranging from 0.9 to 680 mM were inoculated with *T. denitrificans* ATCC 23642 and incubated for 30 d at room temperature in the dark. Sterile medium controls containing the matching Cl^- concentrations were incubated with these cultures. The controls and cultures, were compared for NO_3^- loss using second derivative UV absorbance and IC analyses. The IC analysis was used to verify NO_3^- results obtained using the second derivative UV absorbance method.

Because of the complexity of the undefined HNRB medium, it was tested with more potential interfering substances than the S8 medium. Some oil field waters have high concentrations of Cl^-, $SO_4^{=}$, and, or NH_4^+, so these ions were evaluated as potential interfering substances in the HNRB medium. In addition, NH_4^+, NO_2^- and N_2O, which are products of nitrate reduction, and acetylene, the blocking agent used in the HNRB medium, were tested individually as potential interfering substances for the second derivative UV analysis. To determine whether substances in the medium could cause interference, tests were done using 1/300 dilutions in distilled, deionized H_2O comparing HNRB medium with and without the individual potential interfering substances. These included 5 mM NH_4^+, 40 mM $SO_4^{=}$, 2.2 mM N_2O and 25% (v v^{-1}) acetylene. Two concentrations (760 and 2400 mM) of Cl^- were tested in medium that contained various NO_3^- concentrations (4.5, 3.4, 2.3 and 1.1 mM). The effects of various proportions of NO_3^- and NO_2^- on the second derivative UV absorbance results were tested by preparing HNRB medium with the following four compositions: 3.4 mM NO_3^- and 1.1 mM NO_2^-, 2.3 mM NO_3^- and 2.2 mM NO_2^-, 1.1 mM NO_3^- and 3.4 mM NO_2^- and 0 mM NO_3^- and 4.5 mM NO_2^-.

2.3 Results

2.3.1 Second derivative UV absorbance spectrum

A UV absorbance spectrum and second derivative UV absorbance spectrum of a 81 µM NO_3^- solution are shown in Figure 2.1. The second derivative UV absorbance was calculated using equation (2.1) with $\Delta\lambda$ of 10 nm. The smooth character of the second derivative curve allowed for choice of the wavelength best suited for NO_3^- analysis.

Second derivative UV absorbance spectra of NO_3^- standards, in distilled, deionized H_2O, showed that the most useful wavelengths for NO_3^- analysis were from 222 nm to 228 nm (Figure 2.1b). Because NO_2^- can interfere in second derivative NO_3^- analysis (Crumpton et al. 1992), second derivative absorbance spectra of NO_2^- standards in distilled, deionized H_2O were obtained. The results showed that the second derivative UV absorbance for NO_2^- passed through 0 near a wavelength of 223 nm, agreeing with the results of Suzuki and Kuroda (1987). The second derivative UV absorbance at 226 nm gave the most useful calibration curves for NO_3^- analysis and the wavelength where NO_2^- would not have a significant effect on the results.

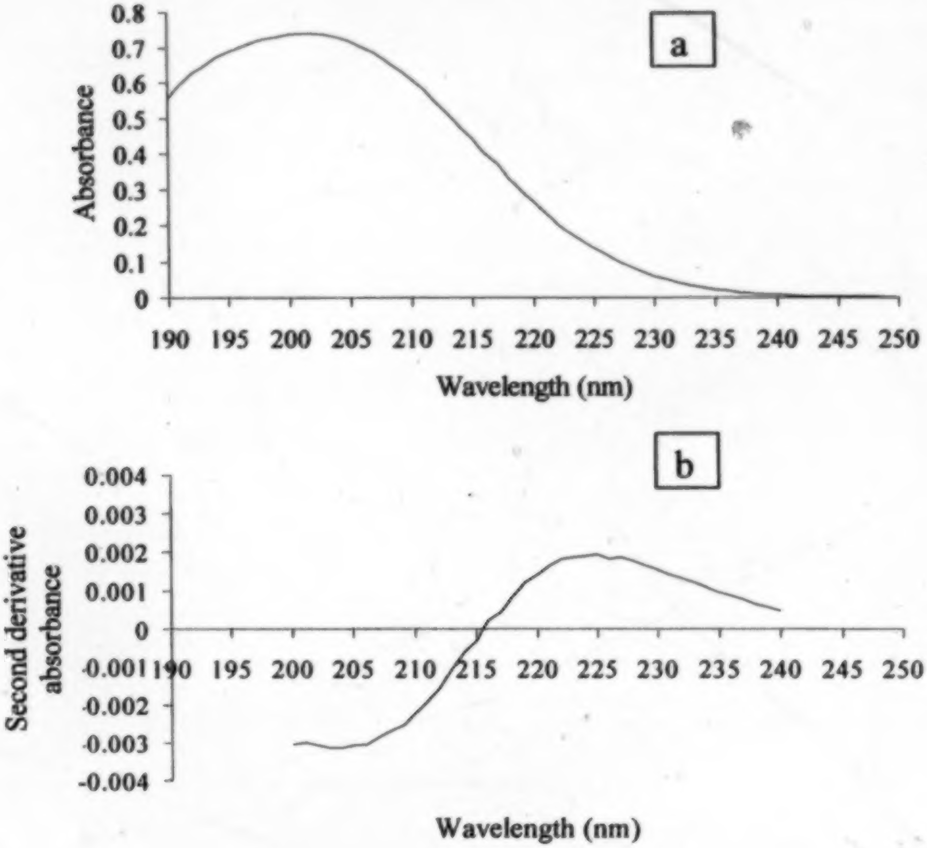

Figure 2.1 Absorbance scan (a) and second derivative absorbance scan (b) of 81 µM NO_3^-. The second derivative was calculated using equation (2.1) and a $\Delta\lambda$ of 10 nm. The smooth character of the curve allowed for choice of the wavelength at 226 nm, which was best suited for NO_3^- analysis.

2.3.2 Second derivative UV absorbance calibration curves for S8 and HNRB media.

Figure 2.2 shows a typical calibration curve for NO_3^- concentrations of 1.6 to 150 µM used for S8 medium. An extended calibration curve was shown to be linear up to 240 µM NO_3^-. The 1/800 dilution of uninoculated S8 medium with 50 mM NO_3^- had a second derivative UV absorbance result of approximately 0.001 at 226 nm. Figure 2.3 shows a

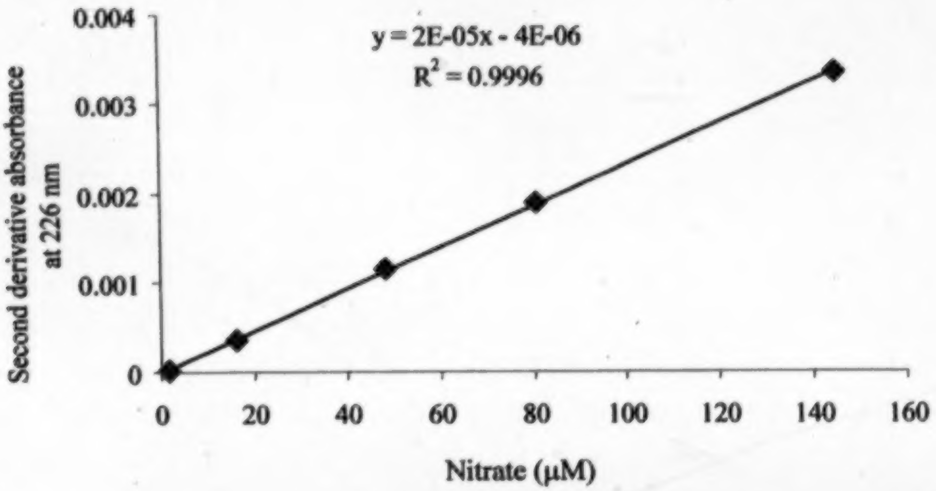

Figure 2.2 Calibration curve for chemolithotrophic NRB medium using KNO_3 to make NO_3^- standards. The reference cell contains distilled, deionized H_2O and the second derivative UV absorbance was taken from calculated values at 226 nm.

typical calibration curve for NO_3^- concentrations of 1.6 to 19 µM used for the HNRB medium with 5 mM NO_3^-. The calibration curve did not pass through the origin, so that second derivative UV absorbance readings were considered below detection when they were lower than the y-intercept for the calibration curve regression formula. A 1/300 dilution of uninoculated HNRB medium typically has a second derivative UV absorbance result of about 0.0007 at 226 nm. Any second derivative UV absorbances lower than the y-intercept value at about 0.0003 would indicate a loss of >57% of the NO_3^- in the medium, which was adequate in determining the presence of NRB in a culture.

2.3.3 Testing for interfering substances in S8 medium

The second derivative UV absorbance values for a 1/300 dilution of S8 medium, with and without NO_3^-, were determined to be 0.0034 and -0.0003, respectively. There appeared to be no obvious component in the medium that gave a significant second derivative UV absorbance reading 226 nm. Thus, it was concluded that there appeared to be no substances in the medium that interfered with the second derivative UV absorbance at 226 nm.

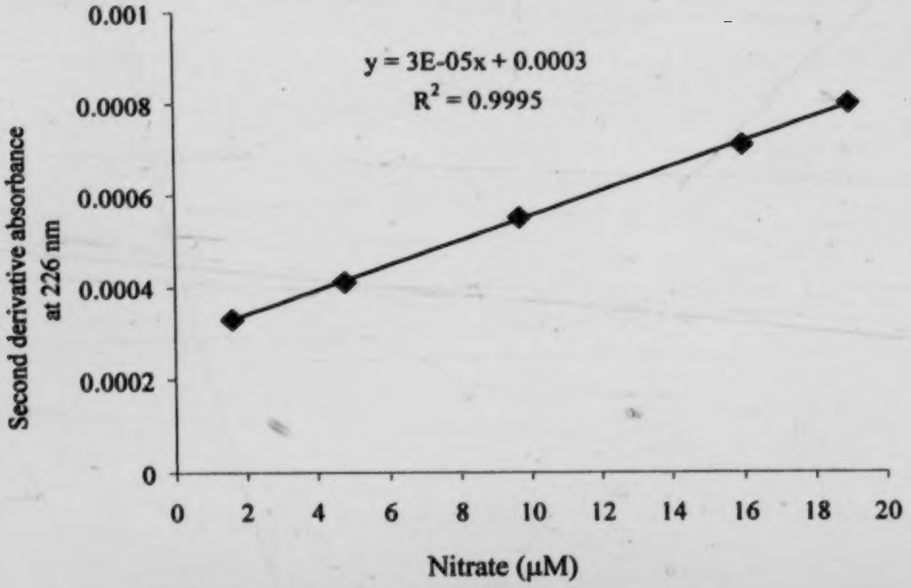

Figure 2.3 Calibration curve for NO_3^- in HNRB medium. KNO_3 was dissolved in a 1/300 dilution of NO_3^--free HNRB medium. The reference cell contained distilled, deionized H_2O, and the second derivative UV absorbance was taken from calculated values at 226 nm.

The NaCl concentrations of the S8 medium were adjusted to match the Cl^- concentrations in the water samples. Table 2.1 summarizes the NO_3^- concentrations in cultures of *T. denitrificans* ATCC 23642 grown in the S8 medium with different Cl^- concentrations. The concentrations of NO_3^- were determined by IC and second derivative UV absorbance. The results in Table 2.1 show good agreement between the two methods and indicate that Cl^- concentrations up to 680 mM did not interfere with the second derivative method. The basis for scoring an MPN tube positive was the consumption of >20% of the NO_3^-. In each case, growth of the *T. denitrificans* over the 30-d incubation time removed 33 to 77% of the NO_3^- based on the second derivative UV analyses. The last column in Table 2.1 shows there was little difference between the percentage of NO_3^- consumed as determined by the two analytical methods. A paired *t*-test was performed on the results for the percentage of NO_3^- consumed using the two analytical methods, and there was no difference between the two methods ($P<0.05$). Thus, the second derivative UV analysis is as reliable as the IC method.

Table 2.1 Comparison of the second derivative UV absorbance values and IC results for a bacterial culture of *T. denitrificans* in S8 medium at different Cl^- concentrations

Cl^- in medium (mM)	Second derivative UV analysis			IC analysis			Difference between NO_3^- consumption comparing IC and second derivative UV (%)
	NO_3^- conc. from second derivative UV analysis[a] (mM)		NO_3^- consumed (%)[b]	NO_3^- conc. from IC analysis[a] (mM)		NO_3^- consumed (%)[b]	
	Bacterial culture	Sterile medium		Bacterial culture	Sterile medium		
0.9	21	44	52	18	44	59	+7
0.9	12	45	73	14	44	68	-5
91	12	45	73	12	44	73	0
100	27	40	33	29	45	36	+3
100	12	40	70	11	45	76	+6
190	11	44	75	11	44	75	0
680	9.8	42	77	9.5	44	78	+1

[a] After 30 d of incubation

[b] Based on a comparison of NO_3^- concentration in growing cultures to NO_3^- concentrations in tubes with sterile medium control after 30 d of incubation.

During the growth of *T. denitrificans* in S8 medium, the nitrate reduction produced NO_2^-, which reached concentrations as high as 1.6 mM. Similarly, the oxidation of thiosulfate by *T. denitrificans* caused the $SO_4^=$ concentration to increase by as much as 45 mM above the original $SO_4^=$ concentration in the medium (1.4 mM). The close agreement between the NO_3^- consumption measured by IC and second derivative UV absorbance (Table 2.1) indicates that the accumulation of NO_2^- and $SO_4^=$ does not affect the second derivative UV measurements.

2.3.4 Testing for interfering substances in HNRB medium

While evaluating methods for preparing a calibration curve for NO_3^- in HNRB medium, it was observed that some component in nutrient broth gave a second derivative UV absorbance at 226 nm. This caused the calibration line to pass through the y-axis above the origin (Figure 2.3). Preparing the NO_3^- standards in diluted NO_3^--free HNRB medium yielded a satisfactory curve.

The second derivative UV absorbance measurements were done on 1/300 dilution HNRB medium with and without 5 mM NH_4^+ and with and without 40 mM $SO_4^=$. The second derivative UV readings were essentially the same in each case, indicating that these ions, which are commonly found in oil field waters, did not interfere with the second derivative UV determination of NO_3^-.

Acetylene was added as a blocking agent to each MPN tube containing HNRB medium. The acetylene caused the accumulation of N_2O in tubes containing gas-producing NRB. These two gases were added individually to the headspaces of tubes containing HNRB medium. After equilibration, second derivative UV determinations of NO_3^- were done, and the determinations showed that neither of these relatively water-soluble gases interfered with the assay.

The possible interference from Cl^- and NO_2^- in HNRB medium were studied in greater detail. Table 2.2 shows the results from testing the effects of Cl^- concentrations of 760 and 2400 mM. The latter was the highest Cl^- concentration in oil field waters that were sampled. Triplicate tests were done on 1/300 dilutions of sterile medium with and without Cl^-, and the mean and standard deviations are shown in Table 2.2. The medium

Table 2.2 Comparison of second derivative UV absorbance values of HNRB medium with different concentrations of NO_3^- and Cl^-

		Second derivative UV absorbance at 226 nm[a]		
NO_3^- (mM)	Cl^- addition (mM)	Without Cl^-	With Cl^-	$P(T\leq t)$[b] two-tail
4.5	760	6.0×10^{-4} ($\pm 5.8 \times 10^{-6}$)	6.3×10^{-4} ($\pm 4.4 \times 10^{-5}$)	0.35
	2400		6.3×10^{-4} ($\pm 1.7 \times 10^{-5}$)	0.06
3.4	760	5.8×10^{-4} ($\pm 2.0 \times 10^{-5}$)	5.8×10^{-4} ($\pm 5.8 \times 10^{-6}$)	0.80
	2400		5.6×10^{-4} ($\pm 3.2 \times 10^{-5}$)	0.35
2.3	760	4.8×10^{-4} ($\pm 5.8 \times 10^{-6}$)	4.7×10^{-4} ($\pm 1.1 \times 10^{-5}$)	0.16
	2400		4.7×10^{-4} ($\pm 1.5 \times 10^{-5}$)	0.15
1.1	760	4.0×10^{-4} ($\pm 5.8 \times 10^{-6}$)	4.0×10^{-4} ($\pm 2.1 \times 10^{-5}$)	1.0
	2400		3.6×10^{-4} ($\pm 1.7 \times 10^{-5}$)	0.009

[a] Samples were diluted 1/300 before analysis. Mean and standard deviation of triplicate analyses are reported.
[b] Probability from *t*-test.

contained four different NO_3^- concentrations, corresponding to 0, 25, 50 and 75% NO_3^- consumption. The second derivative UV absorbances of the medium with and without Cl^- were compared by the *t*-test (Table 2.2). In all but one case, the results were indistinguishable, indicating that the presence of high Cl^- concentrations did not affect the second derivative UV absorbance readings. The only exception was the combination of the lowest NO_3^- concentration (1.1 mM) and the highest Cl^- concentration (2400 mM) tested. This medium contained a molar ratio of Cl^- to NO_3^- of about 2200, and the second derivative UV absorbance reading was slightly less ($P<0.05$) than the reading with no Cl^-. These results demonstrated that the second derivative UV absorbance method was quite insensitive to high Cl^- concentrations.

The growth of NRB produces NO_2^- as an intermediate or end product. Sterile HNRB medium was prepared with different concentrations of NO_3^- and NO_2^-, corresponding to 25, 50, 75 and 100% NO_3^- consumption. The second derivative UV absorbance readings from these solutions were compared to those of medium with the corresponding NO_3^- concentrations but devoid of NO_2^- (Table 2.3). The means of triplicate analyses of 1/300 dilutions of these preparations were compared by the *t*-test. The second derivative UV absorbance readings of medium that contained mixtures of NO_3^- and NO_2^- were not significantly different ($P<0.05$). The final line in Table 2.3 shows that the 1/300 dilution of the medium containing no NO_3^- and 4.5 mM NO_2^- gave a second derivative UV absorbance which was less than the y-intercept of the calibration curve for NO_3^-. The results in Table 2.3 show that the presence of NO_2^- in the HNRB medium did not interfere with the NO_3^- analysis by second derivative UV absorbance.

2.3.5 Comparing MPN results using different methods to detect positive tubes

No indigenous chemolithotrophic thiosulfate-oxidizing NRB were cultured from any of the water samples in S8 medium. However, HNRB were commonly found in the water samples examined in this study. Figure 2.4 shows the MPN results obtained for two oil field water samples and a river water sample using different methods of scoring the tubes positive to provide a MPN index. These methods included turbidity, second

Table 2.3 The effect on second derivative UV absorbance of HNRB medium with various proportions of NO_3^- and NO_2^-

		Second derivative UV absorbance at 226 nm[a]		
NO_3^- (mM)	NO_2^- (mM)	Without NO_2^-	With NO_2^-	$P(T \leq t)$[b] two-tail
3.4	1.1	5.8×10^{-4} ($\pm 2.1 \times 10^{-5}$)	5.8×10^{-4} ($\pm 3.6 \times 10^{-5}$)	0.90
2.3	2.2	5.2×10^{-4} ($\pm 2.5 \times 10^{-5}$)	5.1×10^{-4} ($\pm 7.1 \times 10^{-6}$)	0.69
1.1	3.4	3.7×10^{-5} ($\pm 5.8 \times 10^{-5}$)	3.9×10^{-4} ($\pm 1.0 \times 10^{-5}$)	0.06
0	4.5	3.3×10^{-4}[c]	3.1×10^{-4} ($\pm 1.0 \times 10^{-5}$)	NA[d]

[a] Samples were diluted 1/300 before analysis. Mean and standard deviation of triplicate analyses are reported.
[b] Probability from *t*-test.
[c] y-intercept from the calibration curve for NO_3^-.
[d] Not applicable.

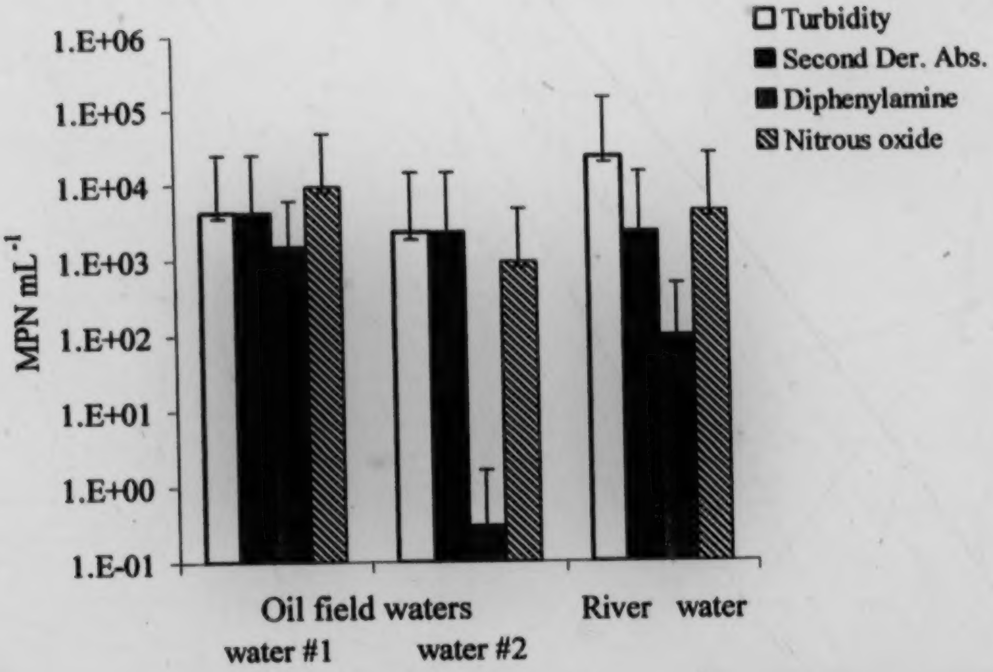

Figure 2.4 Comparisons of MPN values in oil field water samples inoculated into HNRB medium. Various methods for determining growth or nitrate-reducing activity were used. The error bars show 95% confidence interval of the MPN values.

derivative UV absorbance, the diphenylamine spot test method for determining residual NO_3^- and measuring N_2O as an indication of gas-producing NRB. For oil field water samples #1 and #2, no statistical differences (using the method of Cochran 1950) were found among the MPN values determined by turbidity, second derivative UV absorbance analyses or N_2O production. In addition, the MPN values determined by second derivative UV absorbance analysis and the diphenylamine spot test were the same for oil field water #1. In contrast, the MPN value determined from the diphenylamine spot test for oil field water #2 was vastly less than the MPN values determined by the other three methods (Figure 2.4). The lower diphenylamine MPN value for oil field water #2 could have been due to residual NO_3^- in the MPN tubes. This is discussed further in Section 2.4. For the river water sample, the MPN values determined by second derivative UV absorbance analysis and N_2O production were the same (*$P<0.05$*), whereas the MPN value based on the diphenylamine test was slightly lower ($P<0.05$) than those determined by the former two methods.

In total, 17 water samples (15 originating from oil fields) were enumerated for HNRB using the four different methods (as illustrated in Figure 2.4) for scoring the tubes positive to provide a MPN index. The results from seven of the water samples produced the same pattern that was observed for oil field water #1. That is, all four methods gave the same MPN value based on the statistical method of Cochran (1950) ($P<0.05$). Two of the samples gave the same pattern as oil field water #2, with the MPN values based on the diphenylamine test being significantly lower than those values determined by the other three methods. Eight of the samples yielded data similar to those observed with the river water (Figure 2.4). Specifically, two of the samples gave results that matched the river water profile (Figure 2.4). Six samples gave results in which the MPN value based on the diphenylamine analysis was lower than the values obtained by the other three methods, and the MPN value based on turbidity was higher ($P<0.05$) than the values obtained by the other three methods. Of course, the formation of turbidity from microbial growth is not specific for NRB in this medium, and the higher MPN value based on turbidity indicated the growth of fermentative bacteria in this rich medium. For each of the 17 samples, the MPN result based on the second derivative UV absorbance analysis and MPN result based on the N_2O analysis were the same ($P<0.05$). The results in Figure 2.4 illustrate the utility of the second derivative UV absorbance method for determining the consumption of NO_3^- in the HNRB medium.

2.4 Discussion

The goal of this work was to develop a fast and reliable method to screen for NO_3^- consumed in MPN cultures of chemolithotrophic NRB and HNRB. The method, described by Crumpton et al. (1992), using second derivative UV absorbance was evaluated for this purpose. Using a $\Delta\lambda$ of 10 nm for equation (2.1) was adequate for obtaining a good signal-to-noise ratio while giving a reasonable amount of resolution as described by Cahill (1979). The derivative calculation was averaged over several wavelength regions in order to obtain the second derivative curve that had a smooth character as shown in Figure 2.1b and was not affected by noise. Smooth curves were not obtained with any $\Delta\lambda$ value lower than 10 nm (data not shown).

Crumpton et al. (1992) stated that the second derivative UV absorbance method is not suitable for seawater or high saline inland waters because of interfering substances. Their work would have focused on relatively low NO_3^- concentrations in saline waters. The media that was used in this study contained high NO_3^- concentrations (5 and 50 mM) and various concentrations of Cl^-. The dilutions of the samples, required to bring the NO_3^- concentrations into the working range of the calibration curves, were most often sufficient to remove any interference from Cl^-. Crumpton et al. (1992) reported that there was no interference by PO_4^{3-}, NO_2^-, HCO_3^-, Fe^{3+}, Cu^{2+} and $SO_4^{=}$. This study showed no interference by NO_2^-, $SO_4^{=}$ or NH_4^+. The other ions were not tested because they are present in low concentrations in the samples. The second derivative UV absorbance method was easily adapted for use with the S8 medium for chemolithotrophic NRB enumeration.

Laboratory studies with *T. denitrificans* strain F, a sulfide tolerant strain of NRB, showed that its growth could control biogenic sulfide production (Sublette et al. 1994; McInerney et al. 1996). Thus, oil field waters were screened for this group of chemolithotrophic NRB but none was detected in any of the samples. The chemolithotrophic NRB that grow in the S8 medium are considered gas-producing NRB (Sublette et al. 1994), indicating that they grow quickly (Mahne and Tiedje 1995) and would be expected to show a large decrease in NO_3^- concentration in the 30-d incubation period. No decrease of NO_3^- in the medium, inoculated with oil field waters, was observed. However, the control bacterium *T. denitrificans* ATCC 23642 grew in the S8 medium at Cl^- concentrations up to 680 mM (Table 2.1), and the second derivative UV absorbance method was as reliable as the IC method for determining NO_3^- consumption during growth of this reference strain. It is likely that thiosulfate-oxidizing NRB would have been detected if they were present at appreciable numbers in the samples that were examined.

Adapting the second derivative UV absorbance analysis for NO_3^- consumption from the HNRB medium was more challenging. Components in the nutrient broth gave a consistent second derivative UV absorbance of about 0.0003 at 226 nm. Implementing the second derivative UV absorbance for the HNRB medium was important because NO_3^- loss from the organic-containing medium could not be analyzed using IC. Experience showed that the MPN results based on the diphenylamine spot test method were often low, as illustrated by oil field water #2 and the river water in Figure 2.4. By using a 1/300 dilution of NO_3^--free HNRB medium as the diluent for the standards, satisfactory and reproducible calibration curves could be obtained as shown in Figure 2.3. Crumpton et al. (1992) stated that dissolved organic matter did not present a problem with the second derivative UV absorbance method unless the absorbance by organics is extremely high. The 1/300 dilution of the sterile heterotrophic medium diluted the nutrient broth to a concentration of about 13 mg L^{-1}. This amount of organic material had no deleterious affect on the second derivative UV absorbance method with NO_3^- concentrations in the range of 1.6 to 19 μM (Figure 2.3).

For any MPN method, there must be some criterion used to score a culture positive for growth. When studying NRB, the depletion of NO_3^- (Ludvigsen et al. 1999; Davidova et al. 2001) or the production of N_2O (Tiedje 1982, Quevedo et al. 1996) or N_2 (Bekins et al. 1999) are commonly used as indicators of growth of NRB in the culture tubes. Davidova et al. (2001) considered tubes to be positive if there was >10% of the NO_3^- consumed by the culture. In the study by Ludvigsen et al. (1999), tubes for nitrate reducers were scored positive once they became depleted of NO_3^-. In the present study, a culture tube was considered positive for NRB growth if there was a NO_3^- decrease in the medium of >20%. This decrease was easily detected by the second derivative UV method. Table 2.2 shows that the growth of *T. denitrificans* in the S8 medium typically consumed >70% of the NO_3^- over the 30-d incubation. Typically, no residual NO_3^- was detected by the second derivative UV absorbance method in the highest dilution in which NO_3^- consumption occurred in all three tubes of the HNRB medium. In the next higher 10-fold dilutions, those tubes that showed NO_3^- consumption were easily scored positive because the concentrations of NO_3^- were typically depleted by >20%.

Nitrous oxide is produced by NRB that are considered denitrifiers (Tiedje 1982) and dissimilatory ammonium-producing, nitrate-reducing bacteria (Tiedje 1988, Bonin 1996). In the present study, any MPN tube that contained >0.05% N_2O in its headspace was scored positive for the growth of NRB that produce N_2O. This criterion was used for the data presented in Figure 2.4. In all 17 samples that were examined, the HNRB results from second derivative UV absorbance were the same ($P<0.05$) as those determined by N_2O production.

Tiedje (1982) recommended using the diphenylamine spot test to determine which tubes should be analyzed by gas chromatography for N_2O. The results from this spot test indicate which cultures depleted their NO_3^- and were most likely to have accumulated N_2O in the presence of acetylene. The diphenylamine test was used to determine which set of tubes were to be analyzed by the second derivative UV absorbance method. As illustrated in Figure 2.4, the MPN values based on the diphenylamine test were often lower than those based on the second derivative UV absorbance method. This was true for six of the 17 samples that were enumerated for NRB. The difference likely exists because the presence of any residual NO_3^- in the culture would give a blue reaction product from diphenylamine, which would mean that the tube was scored negative. However, if the same tube was analyzed by the second derivative UV absorbance method and showed, for example, >50% of the NO_3^- was consumed, it would be scored positive. The differences in the MPN values derived from these two methods for NO_3^- analysis suggests that the criterion of "complete" removal of NO_3^-, as determined by the diphenylamine test, may be too stringent, resulting in falsely low MPN values in some cases.

The second derivative UV absorbance method is an attractive procedure for determining NO_3^- consumption in media used for enumerating NRB. The high original concentrations of NO_3^- in the media necessitates dilutions to get the NO_3^- concentration in the range of the calibration curves. These dilutions reduce interferences from other components in the cultures and residual medium. This method is most conveniently used to analyze those culture tubes that have shown no reaction in the diphenylamine test and the tubes in the next few higher 10-fold dilutions. The second derivative UV absorbance method is particularly useful for analyzing medium with high organic content that would damage expensive IC analytical columns. In addition, when analyzing thiosulfate-containing medium (e.g. S8 medium), the second derivative UV absorbance method yields results more quickly than the IC method because thiosulfate has a long retention time on an IC column. It was estimated that samples from 20 MPN culture tubes containing thiosulfate-containing medium can be analyzed using the second derivative UV absorbance method in about 3.5 h by an experienced worker. Preparation of the same samples for IC analyses would take about 3 h of the worker's time and an additional 10 h instrument time.

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3. Planktonic nitrate-reducing bacteria and sulfate-reducing bacteria in some western Canadian oil field waters*

3.1 Introduction

Relatively few studies of oil field microbiology have considered nitrate-reducing bacteria (NRB). Indeed, no mention of these bacteria was made in a review of the microbiology of petroleum reservoirs (Magot et al. 2000). NRB can be classified on the basis of the electron donors that they use. They can be chemolithotrophs (autotrophs) that use inorganic compounds, such as sulfide, thiosulfate or FeS as electron donors (Kuenen 1989, Sublette et al. 1994, Gevertz et al. 2000) or chemoorganotrophs (heterotrophs) that use organic compounds as electron donors (Zumft 1992).

For decades, the petroleum industry has been plagued by H_2S produced from the reduction of $SO_4^=$ by sulfate-reducing bacteria (SRB) (Davis 1967, Iverson and Olson 1984). Hydrogen sulfide causes many problems including souring of gas and oil, corrosion of metals and plugging of reservoirs by forming precipitates which reduce the oil recovery (Cord-Ruwisch et al. 1987, McInerney et al. 1993, Rueter et al. 1994, McInerney and Sublette 1997). Of course, H_2S is very toxic.

As the pressure in an oil reservoir decreases, enhanced recovery methods are required to maintain oil production. Waterflooding is a commonly used enhanced recovery method in which source water, comprised of surface water or groundwater, is injected into the reservoir to help drive the oil to the producing wells. Aboveground, the oil is separated from the produced water, and this water, along with source water, is injected back into the reservoir. Waterflooding often stimulates the activities of SRB by introducing these bacteria and, or $SO_4^=$ into the oil field. As a result, a "sweet" crude oil, which has no H_2S, may become a lower-value "sour" crude because of the presence of microbially-produced H_2S.

Biocides are often added to the produced waters and injected into oil reservoirs to curtail detrimental microbes (Boivin 1995). Unfortunately, biocides are not always effective nor do they have long-term inhibitory effects. In some cases, regrowth of unwanted bacteria doubled or tripled after the removal of biocides (Reinsel et al. 1996).

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The addition of NO_3^- to anaerobic wastewater (Poduska and Anderson 1981, Jenneman et al. 1986a), oil wastes from ships (Londry and Suflita 1999) and oil field produced waters (McInerney et al. 1996, Davidova et al. 2001) has stopped sulfide formation. If heterotrophic NRB (HNRB) and SRB are present in these sulfate-containing waters, the addition of NO_3^- establishes a competition between these two groups of bacteria. For a given electron donor, the energy gained from nitrate reduction is greater than the energy obtained from sulfate reduction (Zumft 1992). For example, based on data from Thauer et al. (1977), the free energy change for the oxidation of acetate by NRB and SRB are shown below.

$$5CH_3COO^- + 8NO_3^- + 3H^+ \rightarrow 10HCO_3^- + 4N_2 + 4H_2O \qquad \Delta G^{o\prime} = -495 \text{ kJ (mol } NO_3^-)^{-1} \qquad (3.1)$$

$$CH_3COO^- + SO_4^= \rightarrow 2HCO_3^- + HS^- \qquad \Delta G^{o\prime} = -47 \text{ kJ (mol } SO_4^=)^{-1} \qquad (3.2)$$

These reactions show that, per mol of electron acceptor, the NRB have a large thermodynamic advantage over the SRB. Thus, in the presence of NO_3^-, the HNRB will be more active and suppress the activities of SRB, thereby eliminating the production of H_2S.

The presence of chemolithotrophic NRB provides two advantages for oil reservoirs that contain H_2S. First, the nitrate-reducing, sulfide-oxidizing bacteria (NR-SOB) are able to gain energy from oxidizing reduced sulfur. In this way, the H_2S is consumed. Second, these bacteria produce a variety of products as they use NO_3^- as a terminal electron acceptor (Gevertz et al. 2000), including NO_2^-, NO, N_2O and N_2 (Mahne and Tiedje 1995). The production of N_2O by NRB has been shown to raise the redox potential of a given environment to such an extent that strict anaerobic bacteria, like SRB, are inhibited (Jenneman et al. 1986b). Inhibiting SRB prevents the production of H_2S. Much of the research to eliminate H_2S in oil fields has centered around using chemolithotrophic NRB. The research involves NO_3^- addition to the reservoir to stimulate existing oil field NRB populations or the addition of NO_3^- and cultivated NRB to the reservoir to eliminate H_2S (Jenneman et al. 1999). HNRB also produce N_2O (Tiedje 1982, Zumft 1992), having the same affect on the redox potential as the NR-SOB.

Patents have been granted for controlling sulfide production in oil fields (Hitzman et al. 1995, Jenneman and Gevertz 1997, Hitzman et al. 1998). Two patents (Hitzman et al. 1995, Hitzman et al. 1998) focused on stimulating HNRB, whereas the other patent (Jenneman and Gevertz 1997) focused on stimulating NR-SOB. Jenneman et al. (1997) demonstrated that the injection of NO_3^- into an oil field in Saskatchewan, Canada substantially reduced sulfide production.

Several studies have enumerated NRB in oil field waters using most probable number (MPN) methods with different media formulations. Most formulations would preferentially but not exclusively culture chemolithotrophs. For example, the medium used by Davidova et al. (2001) contained only inorganic compounds except for yeast extract, with thiosulfate serving as the electron donor. The medium used by Telang et al. (1999) contained only inorganic compounds except for acetate, with sulfide serving as the electron donor. Other investigations used sulfide as the electron donor with filter-sterilized produced water from the oil field that was being studied (Gevertz et al. 1995, Jenneman et al. 1997, Telang et al. 1997). The filtered produced waters undoubtedly contained some dissolved organic compounds. The only enumerations of HNRB in produced water appear to be those of Adkins et al. (1992) who used molasses and sucrose as electron donors in their medium.

There appears to be no study that specifically enumerated different nutritional types of NRB in oil field waters, although both chemolithotrophic NRB and HNRB have been implicated in controlling sulfide production. Most notably, there is a lack of information on the presence of heterotrophs. Some workers (Hitzman and Sperl 1994, Mueller et al. 1998) have focussed on the abilities of HNRB to consume volatile fatty acids, such as acetate, propionate and butyrate, which are commonly found in produced waters (Magot et al. 2000). However, recent studies have demonstrated that many hydrocarbons, such as benzene (Burland and Edwards 1999), toluene, ethylbenzene, *m*-xylene, naphthalene and C_6 to C_{12} alkanes can be degraded by HNRB [see Widdel and Rabus (2001) for review]. Many of these hydrocarbons will dissolve in produced waters that are in contact with petroleum and provide carbon and energy sources to stimulate HNRB in nitrate-amended oil fields.

The study presented here was designed to selectively enumerate planktonic HNRB and chemolithotrophic NRB in waters from five oil fields in western Canada and to determine the relative abundances of the different types of NRB. MPN procedures were used to enumerate NRB and SRB, and both of these types of bacteria were detected in most of the oil field water samples. The numbers of planktonic bacteria that could grow aerobically on spread plates [reported as colony forming units (CFU)] were also determined. These numbers and the numbers of SRB tended to increase in the aboveground facilities in the oil fields.

3.2 Materials and methods

3.2.1 Sampling sites

Five oil fields in western Canada were sampled during this study (Table 3.1). Four of the sites were in Alberta: one near Edmonton (A), one near Drayton Valley (B) and two near Stettler (P and N); and one oil field was in Saskatchewan (C). Oil field P was sampled on two occasions, and the samples are designated Pa and Pb (Table 3.2).

At a typical waterflooding oil field site, an emulsion of oil, gas and water reach the surface at the producing wells. Some samples were taken directly at the wellhead, and other samples were taken from a satellite, which is a collecting point for several producing wells. These locations were assigned sample code 1 (Table 3.2), and the locations for the sample codes are shown in Figure 3.1. The gas, oil and water then flow from several satellites to the oil field battery where the emulsion is broken, and the three components are separated using heat and, or gravity at the free water knock out (FWKO) or treater facilities (sample code 2, Table 3.2). The gas and oil are shipped off-site for further processing, and the separated produced water is piped to a storage tank or preinjection site (designated sample code 3, Table 3.2) prior to being pumped via an injection well into the reservoir. Source water (sample code 4, Table 3.2) may be added to the storage tank location and then sent to the oil field or injected directly into the formation.

Biocides, scale and corrosion preventors are used in some of the fields that were sampled. Oil fields A and P did not use biocides at the time of sampling. Operators at oil fields B and N turned off the biocides feed 1 week prior to sample collection so the samples would not be influenced by these chemicals. Because of problems associated with SRB, the operators of oil field C were not willing to stop injection of the biocides, and the sample was collected while biocides were being added.

Only one sample was collected for chemical and bacterial analyses from the site near Edmonton (A3, Table 3.2). This came from the pre-injection line. Similarly, only one sample was collected from the Saskatchewan field. This was from the FWKO (C2). For the sites near Drayton Valley and Stettler, four oil field water samples were collected on each sampling trip (Table 3.2).

4

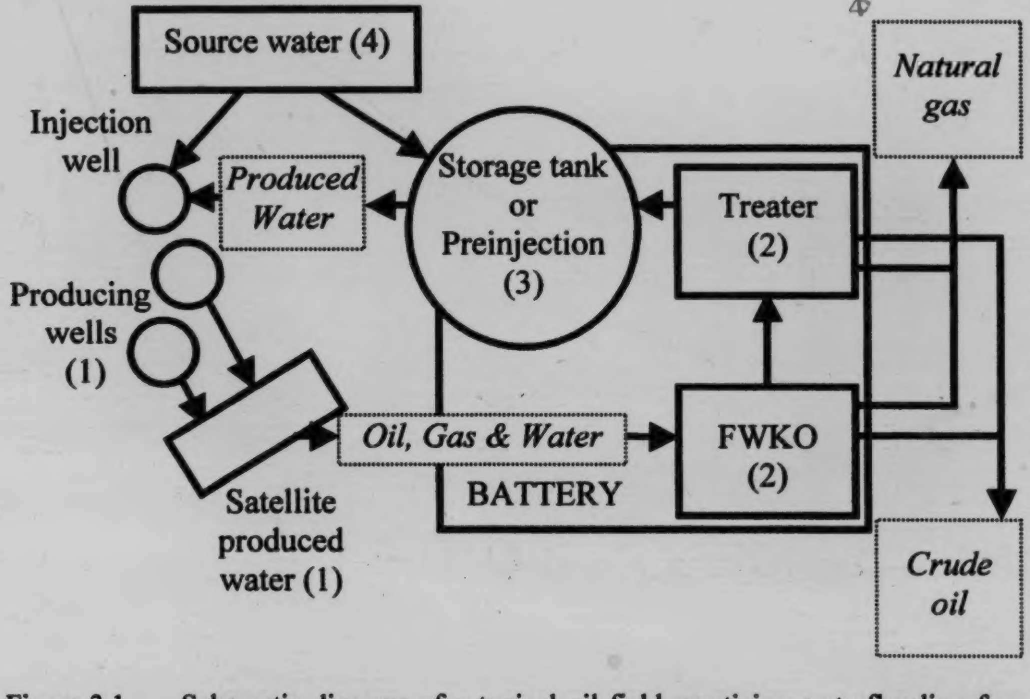

Figure 3.1 Schematic diagram of a typical oil field practicing waterflooding for oil recovery. The numbers in parentheses are part of the sample codes used in Table 3.2 and Figures 3.2, 3.3 and 3.4. FWKO is the free water knock out unit.

Table 3.1 Some characteristics of the five western Canadian oil fields that were sampled during this study

	A	B	C	N	P
Nearest town or city	Edmonton, Alberta	Drayton Valley, Alberta	Coleville, Saskatchewan	Stettler, Alberta	Stettler, Alberta
Production started in	1950	1955	1951	1992	1994
Oil-bearing formation	D3A Leduc	Cardium and Belly River	Bakken	Glauconitic[a]	Glauconitic
Field depth	1520 m	1420 m	810 m	1400 m	1300 m
Production wells in oil field	98	45	245	40	38
Water injection wells in oil field	2	10	110	7	4
Waterflooding started in	1957	1963	1958	1994	1994
Origin of source water	North Sask.[b] River	North Sask. River	Belly River aquifer	Belly River aquifer	Belly River aquifer
Proportion of water in the oil-water emulsion	95%	80-85%	95%	55%	95%
Sampling dates	July, 2000	Sept., 2000	July, 2001	May, 2001	Dec., 2000 Feb., 2001

[a] Also referred to as the Upper Mannville formation (Davidova et al. 2001)

[b] Sask. = Saskatchewan

Table 3.2 Summary of sample locations within oil fields and some physical, chemical and bacterial characteristics of the oil field waters

Oil field	Sample location	Temperature (°C)	pH	Sulfide (mM)	$SO_4^{=}$ (mM)	Cl^- (mM)	Aerobic plate count (CFU mL^{-1})	SRB (MPN mL^{-1})	HNRB (MPN mL^{-1})	NR-SOB (MPN mL^{-1})	Sample code
A	Storage tank	25	7.0	0.3	8	2400	<10	4.3	4.3	ND[a]	A3
B	Wellhead PW[b]	26	8.0	<0.08	<0.005	70	<10	0.9	2.3	<0.3	B1
	Treater	14	7.5	<0.08	0.005	120	15000	150	4300	<0.3	B2
	Storage tanks	14	8.0	<0.08	0.005	110	2900	75	930	<0.3	B3
	Source	19	7.0	<0.08	0.4	0.1	15000	2.1	43	<0.3	B4
C	FWKO[c]	ND	8.5	3	0.6	110	650	930	430	210000	C2
N	Wellhead PW	20	8.0	0.2	8	700	<10	<0.3	<0.3	<0.3	N1
	FWKO	20	8.0	0.3	4	540	840	230	93	93	N2
	Storage tanks	22	8.5	0.9	4	500	28000	2300	23000	93	N3
	Source	13	7.0	<0.08	<0.005	200	4500	43	2300	<0.3	N4
Pa	Satellite PW	24	7.5	2	6	170	<10	2.3	<0.3	<0.3[d]	Pa1
	FWKO	30	7.5	<0.08	4	700	250	750	1.5	<0.3[d]	Pa2
	Preinjection	29	7.5	0.8	6	760	1400	2300	7.5	1500[d]	Pa3
	"Source"[e]	12	9.0	3	13	160	210000	23000	1500	4300[d]	Pa4
Pb	Satellite PW	23	8.5	1	0.3	270	<10	9.3	2.3	<0.3	Pb1
	FWKO	32	8.0	0.5	4	620	20	230	43	<0.3	Pb2
	Preinjection	28	8.0	1	4	500	3200	930	2300	93	Pb3
	"Source"[e]	22	9.0	5	12	200	420	93	1.5	930	Pb4

[a] ND, Not determined.
[b] PW, Produced water.
[c] FWKO, Free water knock out.
[d] The formation of NO_2^- indicated positive MPN tubes.
[e] These "source" waters were actually produced waters from another oil field. See text for details.

3.2.2 Sample collection and chemical analyses

Oil field water samples were collected by completely filling sterile, 4-L plastic bottles. The samples were taken immediately to a work area in the field. As quickly as possible, the water samples were tested for: temperature; pH, using color pHast indicator strips (EM Science, Gibbstown, NJ); and sulfide, using the methylene blue method (CHEMetrics Inc., Calverton, VA). To minimize exposure of the collected water samples to O_2 during transport, portions of the samples were transferred into sealed, sterile 158-mL serum bottles that had previously been made anoxic with O_2-free nitrogen. The transfer was done using a hand pump to create a slight negative pressure in the serum bottle. A piece of sterile tubing was attached to the vacuum pump. The tubing had a sterile needle attached at the other end. A second piece of sterile tubing, with a sterile needle on one end and a sterile pipette attached to the other end, was used to transfer the sample to the serum bottle. The pipette was lowered into a 4-L bottle that contained the water sample. Then, the two needles were simultaneously inserted through the stopper in the serum bottle, and the vacuum pump was used to pull the water sample into the serum bottle. A reduced pressure was maintained using the hand pump until the serum bottle was filled.

In addition, about 100 mL of oil field water was filtered using 0.2 μm pore-size Millex-GS Millipore filters (Bedford, MA) for ion chromatography (IC) analysis, and all samples were packed on ice before being transported to the University of Alberta in Edmonton. Sulfate, NO_3^- and Cl^- were determined by IC (Chapter 2 and Eckford and Fedorak 2002).

3.2.3 Bacterial enumeration and MPN culture analyses

Media for enumerations were inoculated within 24 h of sample collection. Dilutions for the MPN and plate count procedures were made to 10^{-11} using 10-fold serial dilutions of the oil field waters in serum bottles with phosphate buffer (10 mM, pH 7.2). O_2-free nitrogen was used to anaerobically prepare the phosphate buffer and flush serum bottles and syringes. The phosphate buffer and all media contained the Cl^- concentration of the oil field water being tested (Table 3.2). Nitrogen-flushed syringes (1 mL) were used to dispense the appropriate oil field water dilutions to the media. Sterile medium controls were prepared by adding 1 mL of sterile phosphate buffer to each type of medium. These controls were used as references to assess growth and chemical changes that occurred in the medium of viable cultures.

The NRB and SRB were enumerated by 3-tube MPN procedures. The inoculated media were incubated for 30 d at room temperature (approximately 21°C) in the dark before being scored for growth. The resulting MPN values were compared by the statistical method of Cochran (1950).

HNRB were enumerated using a nutrient broth-nitrate medium (HNRB medium) (Section 2.2.1 and Eckford and Fedorak 2002) in sealed 16 x 125 mm Hungate type anaerobic culture tubes (Bellco Glass Inc., Vineland, NJ) with air in the headspace (Tiedje 1982). Acetylene (25% v v^{-1} of headspace gas) was added to each tube to block nitrate reduction at N_2O (Tiedje 1982). After incubation, the growth of NRB and other bacteria produced turbidity in the culture tubes. To verify that growth in the cultures was due to the presence of HNRB, the medium was tested for NO_3^- loss using a second derivative UV absorbance method (Chapter 2 and Eckford and Fedorak 2002). When a decrease of >20% of the NO_3^- concentration in the culture medium was observed, the MPN tube was considered positive for growth of NRB. This threshold was easily detected by the second derivative UV method and it was large enough to ensure that the variability in the NO_3^- concentrations among culture tubes with no growth would not lead to some tubes being falsely scored positive (Chapter 2 and Eckford and Fedorak 2002). Cultures were also analyzed for NO_2^-, using sulfanilamide and *N*-(1-naphthyl)-ethylenediamine dihydrochloride (Clesceri et al. 1998) and for N_2O production (Fedorak et al. 2002). *Pseudomonas stutzeri*, a known denitrifying bacterium (Zumft 1992), was used as a positive control in the HNRB medium.

Chemolithotrophic NRB were enumerated using two types of media to cultivate sulfide-oxidizing or thiosulfate-oxidizing bacteria. Both media were prepared anaerobically in sealed Hungate type anaerobic culture tubes. Thiosulfate medium was modified ATCC 295, S8 medium (ATCC 2000) used to cultivate Thiobacilli (Chapter 2 and Eckford and Fedorak 2002). It will be referred to as S8 medium in this study.

Inoculated medium with *Thiobacillus denitrificans* (ATCC 23642) was used as the positive control for growth. After 30 d of incubation, MPN tubes that had >20% of the NO_3^- consumed from the S8 medium (as determined by the second derivative UV absorbance method described in Chapter 2 and Eckford and Fedorak 2002) were scored positive for thiosulfate-oxidizing, nitrate reducers. Cultures were also analyzed for NO_2^-.

To enumerate NR-SOB, the CSB medium (Telang et al. 1999) was modified by omitting the acetate in order to make it selective for strict chemolithotrophic NRB that oxidize sulfide. The medium (pH 7.5) contained per liter: 0.027 g KH_2PO_4, 0.68 g $MgSO_4 \cdot 7H_2O$, 0.24 g $CaCl_2 \cdot 2H_2O$, 0.02 g NH_4Cl, 0.13 g $(NH_4)_2SO_4$, 1.9 g $NaHCO_3$, 1.0 g KNO_3, 1 mg resazurin and 50 mL of trace elements per liter solution (Telang et al. 1999). The medium was dispensed into Hungate type anaerobic culture tubes, sealed and autoclaved. After cooling, 0.25 mL of a 0.1 M $Na_2S \cdot 9H_2O$ was injected into each tube to give a final sulfide concentration of 2.5 mM as used by Telang et al. (1999). Then acetylene was injected into each tube to give 25% ($v\ v^{-1}$) in the headspace to block nitrate reduction at N_2O. The sealed, inoculated MPN tubes from the first two oil field samplings were incubated on the laboratory bench. There was concern that some of the septa on the Hungate type anaerobic culture tubes might leak and allow O_2 into the tubes (thereby oxidizing the medium). Therefore, the sealed inoculated tubes from the final three oil field samplings were incubated in a Coy anaerobic chamber (Coy Laboratory Products Inc. Ann Arbor, MI) filled with 5% CO_2, 10% H_2 and balance N_2. This ensured that the change in the redox indicator, resazurin, and loss of sulfide was not due to O_2 contamination. The cell yields of the NR-SOB in the modified CSB medium were so low that turbidity could not be observed. The reduced (colorless) medium turned pink due to oxidation of resazurin by the microbially-produced N_2O (Telang et al. 1999). All MPN tubes with modified CSB medium that turned pink were scored positive. Strains CVO and FWKO B, that are known NR-SOB (Gevertz et al. 2000), were obtained from Dr. G. Voordouw's laboratory (University of Calgary) and used as positive controls for the modified CSB medium.

Sulfide was the limiting substrate in the modified CSB medium, so complete consumption of sulfide provided strong evidence of NR-SOB in the MPN culture. An alkaline sodium nitroprusside spot test (Feigl and Anger 1972) was used to detect sulfide in the cultures. Cultures in the modified CSB medium were also analyzed for NO_2^- and N_2O production.

SRB were enumerated using the method of Fedorak et al. (1987). The medium, in tubes with Kaput® closures (Bellco Glass Inc.), contained lactate as the growth substrate, 1 mg L^{-1} resazurin and two iron finishing nails in each tube. Tubes in which the nails turned black from the formation of FeS precipitate were scored positive for growth of SRB.

To determine the numbers of bacteria in the oil field waters that would grow under aerobic conditions, 0.1 mL of the dilutions prepared for the MPN procedure were inoculated to triplicate R2A (Becton Dickinson, Sparks, MD) agar plates. The plates were incubated at approximately 21°C in the dark. After a 7-d incubation period, the plates were examined for growth, and triplicate plates, at the dilution giving bacterial numbers between 30 and 300 colonies, were used for estimations of bacterial numbers.

3.3 Results

3.3.1 Evaluation of counting methods and absence of thiosulfate-reducing bacteria

The criteria used to score MPN tubes for growth of NRB were established for the HNRB and the NR-SOB. For the former group, either the loss of NO_3^- from the medium or the production of N_2O was a candidate for the criterion used to score for growth of NRB. When the denitrifier *P. stutzeri* grew in this medium, it consumed NO_3^- and produced N_2O, as expected. After 30 d incubation of the medium inoculated with oil field water samples, both of these parameters were measured, and they were individually used to determine the MPN values. For each of the 16 samples that yielded growth in the HNRB medium, the MPN results based on the consumption of NO_3^- and the MPN results based on the N_2O analysis were the same ($P<0.05$) (Section 2.2.1 and Eckford and Fedorak 2002). The enumeration results for NRB and SRB are shown in Table 3.2.

The medium used for enumerating HNRB was the medium recommended by Tiedje (1982) for enumerating denitrifying bacteria (modified to contain only one-half the amount of nutrient broth, see Section 2.2.1). Because denitrification is a facultative trait (Zumft 1992), the medium was not prepared using anaerobic methods. Thus, over a 30-d incubation time, growth in the medium could have been a succession of aerobes, facultative anaerobes and even microaerophilic or aerotolerant anaerobes. In addition to measuring NO_3^- loss and N_2O production, the MPN tubes were also scored for growth based on turbidity. Figure 3.2 compares the HNRB medium for the numbers of HNRB based on NO_3^- consumption, with the numbers of bacteria based on growth as indicated by the production of turbidity. If the counts generated by the two methods were the same, the data points fell on the solid, equivalence line shown in Figure 3.2. Those counts that were indistinguishable based on the Cochran statistical method ($P<0.05$), fell within the parallel dashed lines. In total, of the 16 samples that showed growth, 11 fell within the dashed lines, indicating that in most cases, the number of HNRB in a given sample was essentially the same as the number of heterotrophs that grew in this medium. In five samples (Pa2, B1, B4, Pa4 and Pb4), the number of HNRB was less than the number of heterotrophs that grew in this medium.

Of course, there is no reason why the MPN values should fall on the equivalence line (Figure 3.2) The figure is simply comparing the numbers of HNRB with the numbers of heterotrophs that grew in the medium. However, given that so many different types of heterotrophic bacteria could grow in this undefined medium, it is interesting that NO_3^- loss was observed in so many of the MPN dilution series. In addition, the consumption of NO_3^- in so many tubes indicates that the MPN values do not include only the strictly aerobic and fermentative bacteria that could grow in this medium.

Several criteria could be used to score the modified CSB medium positive for growth of NR-SOB which carry out the following reaction (Telang et al. 1999):

$$5HS^- + 2NO_3^- + 7H^+ \rightarrow 5S^0 + N_2 + 6H_2O \qquad \Delta G^{o\prime} = -491\ \text{kJ}\ (\text{mol}\ NO_3^-)^{-1} \qquad (3.3)$$

The criteria include the depletion of sulfide from the medium (which contained a 4-fold molar excess of NO_3^-); the change in the medium from colorless to pink due to the oxidation of the redox indicator, resazurin, by the production of the intermediate N_2O

(Jenneman et al. 1986b, Telang et al. 1999); the formation of NO_2^- as an intermediate of nitrate reduction and the accumulation of N_2O in the headspace gas.

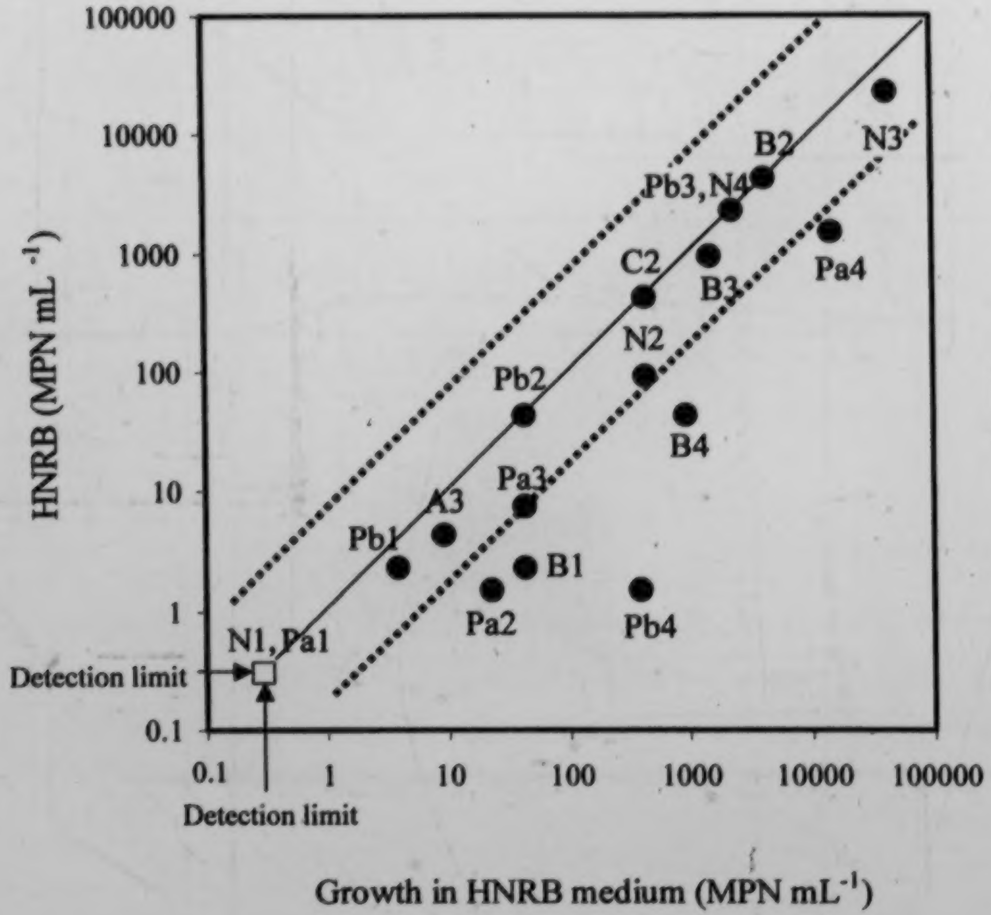

Figure 3.2 Comparison of numbers of HNRB, as determined by NO_3^- consumption, and growth, as determined by turbidity, in HNRB medium. Each point is designated by the sample code given in Table 3.2. Data from the samples in which the counts were below the detection limit are plotted as an open square. Counts that are equal, fall on the solid equivalence line. Those data that fall between the parallel dashed lines have MPN values that are indistinguishable from each other by the statistical method of Cochran (1950) (*$P<0.05$*).

The use of the MPN method for NR-SOB evolved as this project progressed. Work with the first samples taken from oil field B showed no indication of color change in the modified CSB medium after incubation. Work with the second oil field samples (Pa) showed some MPN cultures turned pink but scoring these tubes as positive did not yield utilizable MPN indices. Thus, the medium in each tube was tested for NO_2^-, and those tubes that contained NO_2^- were scored positive. The NO_2^- analysis was used as the basis for MPN results given for the Pa samples in Table 3.2.

MPN cultures in the modified CSB medium for the last three oil field samplings (Pb, C, and N) were incubated in an anaerobic chamber to ensure that O_2 contamination would not cause the redox indicator to oxidize, as was suspected for sample Pa. After incubation, any color change of the MPN cultures for these three samplings was noted, and the medium was assayed for NO_2^- and sulfide. The MPN values were then determined with each individual parameter. No evidence of NR-SOB activity was observed in four of these samples (N1, N4, Pb1 and Pb2, Table 3.2). That is, for N1, N4 and Pb1 the CSB medium showed no color change. For Pb2, the redox indicator in the CSB medium turned pink in the lowest dilution of the MPN series. These tubes were tested further. One milliliter from each pink MPN tube was inoculated to fresh nutrient broth-nitrate and CSB media. Ten-fold dilutions to 10^{-3} were made using these inoculated tubes. After a 30-d incubation, there was turbidity and N_2O production in the 10^{-3} dilution tubes for the nutrient broth-nitrate medium and no change in the 10^{-3} dilution tubes for the CSB medium. The use of the spot test for measuring sulfide precluded the detection of small decreases in sulfide concentration that might have occurred by biotic or abiotic reactions in the medium.

For the remaining five oil field samples (C2, N2, N3, Pb3 and Pb4), the NR-SOB MPN values obtained by measuring the loss of sulfide were the same as those determined by the color change of the medium ($P<0.05$). Four (samples N2, N3, Pb3 and Pb4) of the five MPN values based on N_2O accumulation in the headspace gas yielded lower MPN values than those based on color change or sulfide loss. These results were consistent with other studies that found that sulfide inhibited acetylene blockage (Dalsgaard and Bak 1992, Bonin 1996). In the fifth case (sample C2), the MPN values based on these two parameters were equal. In addition, for the same four samples, the MPN values based on the detection of NO_2^- in the medium were the same as those based on sulfide consumption and pink color formation. Using the criterion of NO_2^- accumulation in the medium to score for MPN values, sample C2 gave a lower MPN (*P<0.05*) using NO_2^- results than when sulfide depletion or oxidation of the redox indicator was used to score the tubes. Thus, scoring positive tubes based on the color change of the resazurin (Jenneman et al. 1986b, Telang et al. 1999) was the easiest procedure, and it agreed completely with the depletion of sulfide from the medium. The NR-SOB strains CVO and FWKO B both grew in the modified CSB medium, consuming sulfide and turning the medium pink.

Although elevated sulfide concentrations are inhibitory to some chemolithotrophic NRB (Sublette and Woolsey 1989), the well characterized NR-SOB, strains CVO and FWKO B, grow at 10 mM and 15 mM sulfide, respectively (Telang et al. 1999). The sulfide concentration in the modified CSB medium was 2.5 mM, which was higher than most sulfide concentrations in the water samples collected (Table 3.2), except "source" waters (Pa4 and Pb4) and C2. Telang et al. (1999) used 2.5 mM sulfide in their CSB medium, and no attempt was made to optimize the sulfide concentration in the modified medium.

There were no thiosulfate-oxidizing NRB cultivated from any of the oil field water samples. However, when each set of oil field samples was inoculated, S8 medium amended with the same Cl^- concentration observed in the oil field water samples was inoculated with *T. denitrificans* (ATCC 23642). The *T. denitrificans* was the positive control for growth. The reference culture grew well in the medium with Cl^- concentrations below 760 mM. In addition, Dr. K.L. Sublette (The University of Tulsa, USA) verified that *T. denitrificans* strain F grew in the S8 medium.

3.3.2 Temperature and pH of oil field waters

None of the oil reservoirs was very hot, and the temperatures of the produced waters from the wellheads and the satellites were between 20 to 26°C (Table 3.2). The source waters were generally colder than the waters from the wellheads or satellites. The highest temperature recorded was 32°C in the FWKO at oil field P (sample Pb2, Table

3.2). The sample from oil field C was taken by the oil field operators and transported to the U of A. No temperature reading was available for this sample.

The pH measurements were done on the samples prior to transferring them to evacuated serum bottles. Exposure of the produced waters to a reduced pressure may have caused a loss of dissolved CO_2 and affected the pH. The pH values of the water samples were generally between 7.0 and 8.5. The only exceptions were the "source" waters from oil field P (sample Pa4 and Pb4, Table 3.2) which were pH 9. These data indicate that neither the temperature nor pH of these waters would adversely affect microbial growth.

3.3.3 Oil field A

This oil field was only a short distance from the laboratory, and it was the first field sampled. Only one sample, from the water storage tank, was taken and this was used to test some methods for this study. Although the sulfate concentration was high (8 mM, Table 3.2), the sulfide concentration was low (0.3 mM), and the operators did not consider the field to be souring. This field had the highest Cl^- concentration (2400 mM) of any that were studied. The number of aerobic bacteria was below the detection limit of the plate count method, and some SRB were detected in the produced water (Table 3.2). The method for enumeration of NR-SOB had not been implemented at that time, but a small number of HNRB (4.3 mL^{-1}) were detected.

3.3.4 Oil field B

The operators at this oil field said that H_2S production occurred in November and May. Sulfide was not detected in any of the four sample locations, and the $SO_4^=$ concentrations were very low in the produced waters. The source water used in this field is from the North Saskatchewan River (Table 3.1), which has a higher $SO_4^=$ concentration (0.4 mM) than the produced waters. SRB were detected in all four samples, with MPN values ranging from 0.9 to 150 mL^{-1} (Table 3.2). No colonies were observed in the aerobic plate count of the wellhead produced water, but bacteria, able to grow aerobically, were abundant in the other three samples, with counts ranging from 2.9×10^3 to 1.5×10^4 mL^{-1}. No NR-SOB were detected in any of the samples from field B, but HNRB were found in each of the samples. The highest HNRB counts were in samples from the treater and storage tanks.

3.3.5 Oil field C

Produced waters from this oil field have been studied extensively (Jenneman et al. 1996, 1997; Telang et al. 1997) because of its severe souring problem and it was the source of novel NR-SOB (Gevertz et al. 2000). The only sample available was from the FWKO, which contained 3 mM sulfide (Table 3.2). Because of the problems caused by microbial activities in this field, the operators were not willing to interrupt the addition of biocides, so these inhibitors were being added at the time of sampling. Nonetheless, all four groups of bacteria were found in the produced water (Table 3.2), with the NR-SOB being the most abundant (2.1×10^5 mL^{-1}).

3.3.6 Oil field N

Over the past few years, souring has become a problem at this field. The sulfide concentrations in the produced waters ranged from 0.2 to 0.9 mM (Table 3.2). The source water for this field is the Belly River aquifer (Table 3.1), which has a very low $SO_4^=$ concentration. As shown in Table 3.2, $SO_4^=$ originates from the oil reservoir. The source water contained SRB, HNRB and bacteria that grew aerobically on plates. However, no NR-SOB were detected in this water. None of the four groups of bacteria were detected in the produced water from the wellhead, but all four groups were detected in the samples from the FWKO and the storage tanks (Table 3.2).

3.3.7 Oil field P

This souring oil field was sampled on two occasions. Normally, the source water for oil field P is also the Belly River aquifer, but for a period of time, this groundwater source was not available. Operators of another oil field in the vicinity trucked produced water to oil field P for disposal by injecting into this field. At both sampling times, the only "source" water being injected into the field was actually produced water from a neighboring oil field. This "source" water contained more sulfide and $SO_4^{=}$ than any of the produced waters from oil field P (Table 3.2). On the first oil field sampling trip (Pa), the "source" water had a very high number of colonies on the aerobic plate count medium (2.1×10^5 mL^{-1}) and high numbers of SRB (2.3×10^4 mL^{-1}). It also contained relatively high numbers of HNRB and NR-SOB (Table 3.2).

No colonies grew on the aerobic plate count medium, and no NR-SOB were detected in either produced water from the satellite location at field P (Table 3.2). Similarly, no HNRB were found in the satellite sample taken on the first trip (Pa), but 2.3 HNRB mL^{-1} were found in the second oil field sampling trip (Pb). Various numbers of SRB and HNRB were found in the FWKO, the preinjection and "source" waters. NR-SOB were present in the preinjection and "source" waters from both sampling times (Table 3.2).

3.3.8 Comparison of SRB numbers

Figure 3.3 summarizes the numbers of SRB (enumerated with lactate as the carbon and energy source) in samples taken from oil fields B, P and N. These MPN values all show a common trend. The lowest numbers of SRB were found at the wellhead (fields B and N) or the satellite (field P) and these ranged from <0.3 mL^{-1} (sample N1) to 9.3 mL^{-1} (sample Pb1). These samples provide the best estimate of the numbers of SRB just as the oil-water emulsions leave the reservoirs. Adkins et al. (1992) also found low numbers of SRB in samples taken as near as possible to wellheads. Their MPN values of SRB were ≤ 5 mL^{-1}.

Figure 3.3 shows that as the waters move through the aboveground handling facilities, such as treaters or FWKO units, the numbers of planktonic SRB increase markedly. For example, in oil field B the number increased from 0.9 mL^{-1} in the wellhead sample (B1) to 150 mL^{-1} in the treater sample (B2), and in oil field P, the number increased from 2.3 mL^{-1} in the satellite produced water sample (Pa1) to 750 mL^{-1} in the

FWKO sample (Pa2). The numbers of SRB in the storage tanks or preinjection waters were essentially the same as those in the treater or FWKO. For instance, the MPN values for samples B2 and B3 (Figure 3.3) were the same ($P<0.05$), and there was no difference between the MPN values for samples Pa2 and Pa3 ($P<0.05$). In oil field N, the number of SRB in the storage tank (N3) was slightly greater than ($P<0.05$) the number in the FWKO (N2).

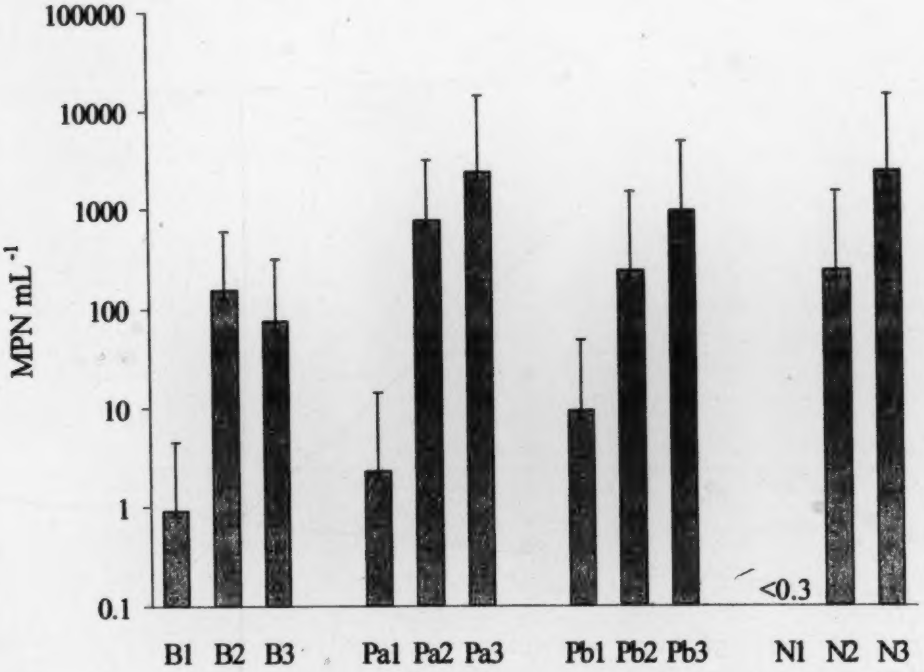

Figure 3.3 SRB counts in various waters from oil fields B, P (sampled on two occasions) and N. The small bars represent the 95% confidence interval of the MPN values. See Table 3.2 for sample codes.

3.4 Discussion

Three of the oil fields studied during this project began operation in the early 1950s and the other two in the early 1990s (Table 3.1). These represent four different oil formations at depths between 810 and 1520 m. All have been operated with waterflooding for many years. The number of production wells varies from 38 to 245, and the number of injection wells varies from two to 110 (Table 3.1). The average water cut (the proportion of water in the oil-water emulsions recovered from the wellheads) varies from 55 to 95%. Thus, more water than oil is being handled at these facilities.

The major focus of this study was to enumerate planktonic NRB from oil field waters. In particular, the aim was to differentiate among the types of NRB present in these fields to assess which type was most abundant and might be stimulated by nitrate amendment to control sulfide production. The enumeration methods differentiated between chemolithotrophic NRB and HNRB in oil field waters.

Laboratory studies with *T. denitrificans* strain F, a sulfide tolerant strain of NRB, showed that its growth could control biogenic sulfide production (McInerney et al. 1992, 1996; Sublette et al. 1994). Thus, the western Canadian oil field waters were screened for this group of chemolithotrophic NRB. However, none was detected in any of the samples, which is consistent with the work of McInerney et al. (1992) who detected no denitrifying thiobacilli in formation water from a gas storage field.

Thiosulfate was the major electron donor in the medium used by Davidova et al. (2001) but, their medium also contained yeast extract. They enumerated NRB in water samples from field N and found these bacteria in each of the six samples they examined. The MPN values were about 100 NRB mL^{-1}. In contrast, using the S8 medium, no thiosulfate-oxidizing NRB were detected in this oil field. The medium in this study was devoid of any utilizable carbon source, which suggests that yeast extract may have been supporting growth of HNRB in the medium used by Davidova et al. (2001).

Adkins et al. (1992) used nitrate-containing media with molasses or sucrose to enumerate HNRB in produced waters from some petroleum reservoirs. Each sample was taken as near the wellhead as possible. They incubated their cultures at 37°C and detected HNRB in each of the five samples they collected. The numbers were very low, and the highest count was 4 mL^{-1}. The results in this study showed that the highest HNRB count for the wellhead or satellite samples was only 2.3 mL^{-1} (Table 3.2), in good agreement with the findings of Adkins et al. (1992).

Figure 3.4 shows a comparison of numbers of planktonic HNRB and NR-SOB in the oil field waters. Any point that appears in the region enclosed by the dashed line had HNRB and NR-SOB counts that were indistinguishable from each other ($P<0.05$). In the

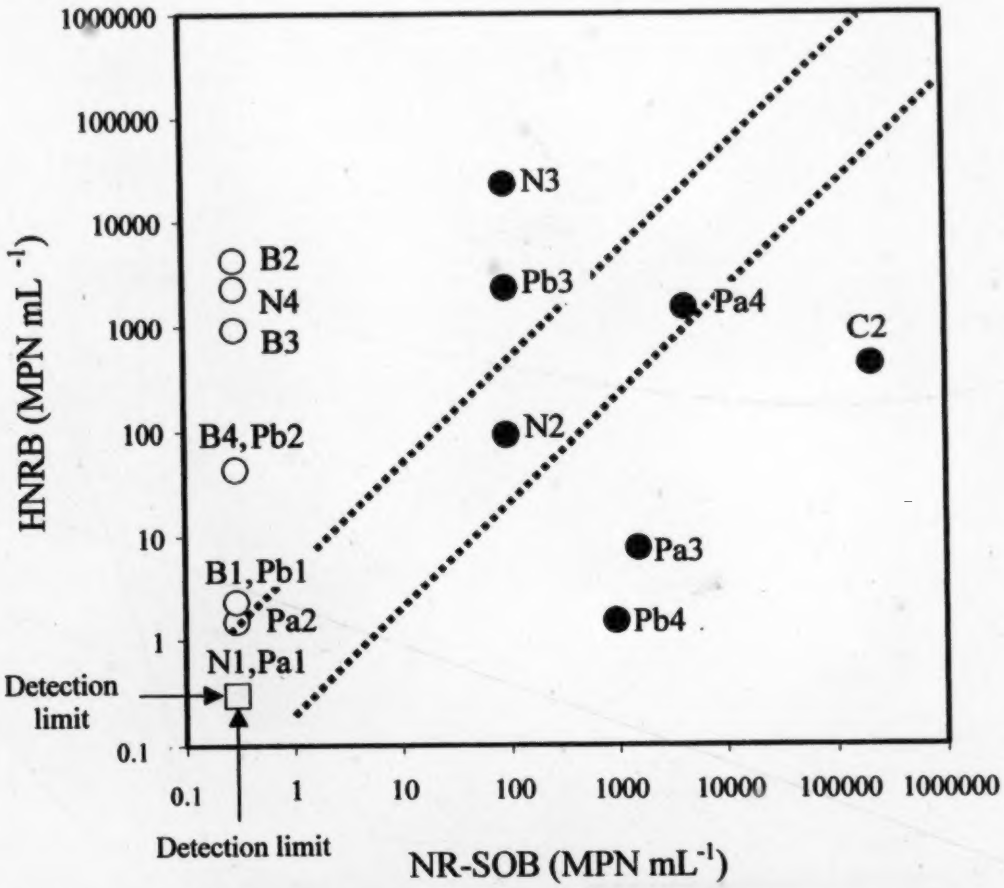

Figure 3.4 Comparison of the MPN values of HNRB and NR-SOB in 17 oil field waters examined in this study. Each point is designated by the sample code given in Table 3.2. Data from the samples in which both HNRB and NR-SOB were below the detection limit are plotted as an open square. Data from the samples in which NR-SOB were below the detection limit, but HNRB were detected, are plotted as open circles. Data from the samples in which both HNRB and NR-SOB were detected are plotted as solid circles. Those data that fall between the parallel dashed lines have HNRB and NR-SOB counts that are indistinguishable from each other by the statistical method of Cochran (1950) (*$P<0.05$*).

cases in which no MPN value could be determined because there was no growth in any of the MPN tubes that contained HNRB medium or modified CSB medium, the value was plotted at 0.3 mL^{-1}, the detection limit of the method. Data from 17 water samples are plotted in Figure 3.4. Three samples (C2, Pa3 and Pb4) contained higher numbers of NR-SOB than HNRB. The MPN values for the NR-SOB and HNRB in three of the samples (Pa4, N2 and Pa2) were equal (*$P<0.05$*). Neither group of NRB was detected in two produced water samples (N1 and Pa1). The remaining nine samples contained higher numbers of HNRB than NR-SOB. In seven of these samples (B1, Pb1, B4, Pb2, B3, N4 and B2), no NR-SOB were detected. The results in Figure 3.4 show that HNRB were more abundant than NR-SOB in 9 of the 15 oil field waters that yielded NRB counts.

The sample that contained the highest number of NR-SOB (C2) was from oil field C. This field has been the focus of several studies by other workers (Jenneman et al. 1997, 1999; Telang et al. 1997; Nemati et al. 2001). In 1996, NO_3^- was injected into a portion of oil field C for 50 d to demonstrate that this amendment could control sulfide production (Jenneman et al. 1997, 1999). Jenneman et al. (1997) supplemented filter-sterilized produced water from this field with NO_3^- and used this preparation as the growth medium for enumerating NRB at various locations in the oil field. Before nitrate injection into the oil field, they found 10^4 to 10^5 NRB mL^{-1} at the injector wells and <10 NRB mL^{-1} at the producing wells. During the period of nitrate injection, the numbers of NRB increased to as high as 10^8 mL^{-1} (Jenneman et al. 1997). Using CSB with acetate, Telang et al. (1999) found 10^6 mL^{-1} NRB in a produced water sample from oil field C.

Due to limited resources, only one sample from oil field C was studied, and this sample was taken while biocides were being injected into the field. The sample came from the FWKO, which was the origin of the novel NR-SOB described by Gevertz et al. (2000). Undoubtedly, the filter-sterilized produced water used by Jenneman et al. (1997) contained dissolved organic compounds, and the CSB medium used by Telang et al. (1999) contained acetate. It is very likely that both HNRB and chemolithotrophic NRB were enumerated in these media. Acetate is known to serve as a substrate for heterotrophic nitrate reduction (Beauchamp et al. 1989). The modified CSB medium used in this study contained no organic carbon and was designed to select for chemolithotrophic NR-SOB. This medium gave a count of 2.4×10^5 NR-SOB in the FWKO produced water (Table 3.2). Using the medium for denitrifying bacteria, there were 430 HNRB mL^{-1} in the FWKO water (Table 3.2). Telang et al. (1997) used the reverse sample genome probe (RSGP) method to monitor the effects of NO_3^- addition to oil field C. Among their reference DNA preparations were "standards" of three heterotrophs that reduced NO_3^- to NO_2^-, and they detected these HNRB in the produced waters from oil field C (Telang et al. 1997). The detection of viable HNRB in this oil field, during the present study, illustrates that the MPN method can detect the same physiological group of NRB that Telang et al. (1997) detected using genome probing methods.

Davidova et al. (2001) studied two oil fields (one of which was oil field N) and reported that the majority of sulfide production appeared to occur after the oil was pumped aboveground, rather than in the reservoir. The distribution of SRB in the oil fields, examined in this study (Figure 3.3), are consistent with that observation (Davidova et al. 2001). The highest sulfide concentration in oil field N (Table 3.2) was found in the storage tank water (sample N3). Oil field P, on the other hand, had high sulfide concentrations in the satellite produced waters (samples Pa1 and Pb1, Table 3.2), possibly an effect of sulfide in the "source waters" (section 3.3.7). The observation for oil field N suggests that the activities of SRB aboveground are increasing the sulfide being reinjected into the reservoir. This sulfide will contribute to souring of the petroleum recovered from these fields. Thus, the aboveground facilities would be potential targets for nitrate amendment to control sulfide production. As shown in Table 3.2, each of the produced water samples collected from the aboveground facilities contained HNRB and, or the chemolithotrophic NR-SOB which would likely be stimulated by nitrate amendment. However, the concentrations of dissolved organic carbon in the produced waters were not determined, so there is no measure of the amounts of potential electron donors for the HNRB that would be stimulated by nitrate amendment.

A plate count method was also used to enumerate heterotrophic bacteria that could be cultivated under aerobic conditions. None were detected (i.e. <10 mL^{-1}) in any of the wellhead or satellite samples (Table 3.2). Adkins et al. (1992) also enumerated heterotrophic aerobes in oil field waters taken as near the wellhead as possible. Using an MPN method, their counts were between 0.1 and 20 mL^{-1} in four of five samples. No aerobes (<0.1 mL^{-1}) were detected in the fifth sample. Thus, none of the wellhead oil field waters examined in the present survey and in the study of Adkins et al. (1992) had very high numbers of bacteria capable of growing under aerobic conditions.

The data in Table 3.2 show that the numbers of bacteria detected by the aerobic plate count increased markedly as the produced waters move through the aboveground facilities. For example, in the oil field N samples, the aerobic counts increased from

<10 mL^{-1} at the wellhead (N1) to 840 mL^{-1} in the FWKO (N2) to 2.8×10^4 mL^{-1} in the storage tanks (N3). This was the same trend as observed for the SRB (Figure 3.3), again illustrating the increase in microbial numbers through the aboveground facilities.

In laboratory studies, Wright et al. (1997) investigated the effects of nitrate amendment to bacteria in four produced brines from west Texas oil fields. Two oil fields had not been subject to extensive waterflooding and all oil fields had reservoir temperatures of 40 to 60°C. The addition of NO_3^- stimulated bacterial oxidation of sulfide in three of the brines. However, the rate of oxidation was increased significantly by the addition of glucose, organic acids (acetate and formate) and vitamins. These results implied that heterotrophic bacteria played a key role in the oxidization of sulfide, although no mechanism was suggested (Wright et al. 1997).

The studies described here have demonstrated that NRB were detected in each of the five western Canadian oil fields that were studied (Table 3.2). In a few cases, these were not detected in the produced waters from the wellheads or the satellites, but planktonic NRB were always found in produced waters from the aboveground operations. Planktonic HNRB were often more abundant than planktonic NR-SOB (Figure 3.4). Of course, the water samples only give "rough measures of the numbers of SRB" and other bacteria because these bacteria are found in biofilms in the oil reservoir and the oil field water system (McInerney and Sublette 1997).

Nitrate amendment to oil field waters provides the potential to stimulate both types of NRB and control sulfide production by SRB. From equation (3.1), the oxidation of acetate by HNRB has a $\Delta G^{o\prime} = -495$ kJ (mol $NO_3^-)^{-1}$. Based on the work of Burland and Edwards (1999), the oxidation of benzene by HNRB has a $\Delta G^{o\prime} = -498$ kJ (mol $NO_3^-)^{-1}$. From equation (3.3), the oxidation of sulfide by NR-SOB has a $\Delta G^{o\prime} = -491$ kJ (mol $NO_3^-)^{-1}$. The energy yields from these reactions are quite similar, so nitrate amendment to produced waters that contain both HNRB and NR-SOB should stimulate both types of bacteria with no competition for electron donors. However, other environmental factors may select for one group or another. These factors can include carbon availability for HNRB, pH, available inorganic nutrients as well as kinetic considerations for the various enzymes used during nitrate reduction. For a discussion on nitrate reductases, see Richardson et al. (2001). To date, there has been little direct evidence that HNRB play a role in controlling sulfide production. However, the results of investigations by Jenneman et al. (1997), Wright et al. (1997), Telang et al. (1999) and Davidova et al. (2001) provide indirect evidence that HNRB may be stimulated when NO_3^- is added to oil field waters.

Although HNRB were detected in the produced waters (Table 3.2), understanding the roles of these bacteria and SRB in produced waters is crucial to assessing the utility of nitrate amendment to control sulfide. Indeed, different scenarios are possible during nitrate amendment to an oil field. For example, the HNRB may out-compete the NR-SOB for NO_3^- needed to oxidize sulfide, thereby hindering sulfide removal. It is also known that some SRB use NO_3^- as an electron acceptor (Cypionka 1995). Pure culture studies with different species of SRB have shown that in the presence of both $SO_4^=$ and NO_3^-, either $SO_4^=$ or NO_3^- may be the preferred electron acceptor, or both electron acceptors can be reduced concomitantly (Cypionka 1995). Thus, the addition of NO_3^- might increase the numbers of SRB in produced waters, which may become problematic after nitrate amendment ceases. However, there was no indication that either of these scenarios occurred in the Saskatchewan oil field studied by Jenneman and co-workers (Jenneman et al. 1997, 1999; Telang et al. 1997).

In summary, planktonic NRB, planktonic SRB and aerobic bacteria were detected in each oil field. Oil fields B, N and P had water samples collected from the satellite or wellhead and aboveground facilities. Sulfate was detected in wellhead or satellite waters for oil fields N and P only. Oil field B wellhead produced water had no detectable $SO_4^=$, there was no $SO_4^=$ in the treater facility or storage tanks for oil field B and the source water had a very low $SO_4^=$ concentration (Table 3.2). These results indicate that $SO_4^=$ used by SRB may have originated from the reservoir for oil fields N and P and that $SO_4^=$ found in oil field B could come from the waterflood.

For all five oil fields there is a potential for sulfide production because SRB and $SO_4^=$ were present. There is a potential for success using NO_3^- to remove sulfide from oil fields C, N and P because NR-SOB were found. Oil fields A, B, C, N and P contained HNRB so there is a potential in these oil fields for HNRB to out-compete heterotrophic SRB and produce intermediate products to control SRB after NO_3^- addition (Table 3.2).

3.5 Literature cited

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4. Chemical and microbiological changes in laboratory incubations of nitrate-amendment "sour" produced waters from three western Canadian oil fields[*]

4.1 Introduction

Sulfate-reducing bacteria (SRB) and their activities in oil fields have been studied for many years. At one time, they were shown to be capable of releasing bitumen from oil sands as well as conventional oil from laboratory test columns, but the detrimental effects of SRB far outweigh any positive contribution they could have in oil fields (Jack 1993). The major detrimental effect is the production of H_2S, which is toxic, causes "souring" of oil and induces corrosion in oil fields. Hydrogen sulfide leads to the production of iron sulfide, which precipitates and reduces oil recovery (Cord-Ruwisch et al. 1987, Jack 1993, McInerney et al. 1993, Rueter et al. 1994, McInerney and Sublette 1997).

Besides SRB, oil reservoirs have diverse and active anaerobic microbial populations (Magot et al. 2000), although there is some doubt whether all of the microbes in an oil field are indigenous (Magot et al. 2000, Spark et al. 2000). However, many oil fields that have been subjected to waterflooding, which re-pressurizes the reservoir by injecting water into the oil-bearing stratum, have many types of bacteria in an ecosystem that allows for the production of H_2S (Magot et al. 2000). The use of drilling mud and the addition of makeup water to the oil reservoir are ways in which $SO_4^{=}$, needed for H_2S production by SRB, is introduced to oil reservoirs (Iverson and Olson 1984).

Because H_2S production is a detriment to oil fields, much effort and expense has been spent to eliminate SRB. The most widely used method for H_2S control is biocide application to the oil reservoir (Boivin 1995, Jack and Westlake 1995). Once bacterial corrosion has been established, high-concentration, long-term biocide treatment is necessary. The amount of biocide required for an oil field waterflood operation, in which produced water is separated and recycled through the oil reservoir, could be more than 100,000 liters per year (Jack and Westlake 1995). Although biocides are useful, they are not always effective in the short- or long-term (Reinsel et al. 1996). An alternative method that has been considered to eliminate H_2S and control the activities of SRB in oil reservoirs is treatment with NO_3^-.

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Studies in which NO_3^- was added to anaerobic wastewater (Poduska and Anderson 1981, Jenneman et al. 1986a,), oily wastes from ships (Londry and Suflita 1999) and oil field produced waters (McInerney et al. 1996, Davidova et al. 2001) have showed that NO_3^- stops the production of sulfide. Nitrate stimulates nitrate-reducing bacteria (NRB) that out-compete SRB for electron donors and produce NO_2^- or N_2O, illustrated below, which increases the redox potential of the environment (above -100 mV) to inhibit the strict, anaerobic SRB.

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \qquad (4.1)$$

Mixed cultures that contained NRB and the redox indicator resazurin turned from colorless to pink when the redox of the medium increased as a result of the accumulation of products from nitrate reduction (Jenneman et al. 1986b, Reinsel et al. 1996, Jenneman and Gevertz 2000). Overall, the main advantage of nitrate reduction by NRB is the formation of end products that are less harmful than H_2S (Zumft 1992).

There are two types of NRB that can be stimulated by the presence of NO_3^-. One is the chemoorganotrophic (heterotrophic) NRB or HNRB that use organic compounds as electron donors. As is illustrated in equations (4.2) and (4.3), using acetate as an electron donor, nitrate reduction yields more energy per mol of terminal electron acceptor than sulfate reduction (Thauer et al. 1977).

$$5CH_3COO^- + 8NO_3^- + 3H^+ \rightarrow 10HCO_3^- + 4N_2 + 4H_2O$$

$$\Delta G^{o\prime} = -495\ kJ\ (mol\ NO_3^-)^{-1} \qquad (4.2)$$

$$CH_3COO^- + SO_4^{=} \rightarrow 2HCO_3^- + HS^- \qquad \Delta G^{o\prime} = -47\ kJ\ (mol\ SO_4^{=})^{-1} \qquad (4.3)$$

Thus, HNRB out-compete heterotrophic SRB for electron donors, thereby suppressing sulfide production. Oil field waters contain dissolved organic compounds including short-chain fatty acid anions, like acetate, propionate and butyrate as well as aromatic compounds, such as toluene and phenols (Barth 1991, McInerney et al. 1993, Sperl et al. 1993, Hitzman and Dennis 1997, Magot et al. 2000) that are substrates for heterotrophs.

The second type of NRB is the chemolithotrophic NRB. Among these are *Thiobacillus denitrificans* and the nitrate-reducing, sulfide-oxidizing bacteria (NR-SOB). If these bacteria are present in the oil field, they will gain energy by oxidizing reduced inorganic sulfur compounds. They are also capable of forming products from nitrate reduction that will raise the redox potential of the environment. As a result, these bacteria not only remove sulfide but also suppress the sulfide formation of the SRB (Jenneman and Gevertz 1997).

Although several laboratory studies (Hitzman and Sperl 1994, Telang et al. 1999, Davidova et al. 2001) and field studies (Gevertz et al. 1995, Jenneman et al. 1997, Telang et al. 1997) have demonstrated that nitrate amendment to oil field waters increases the numbers of NRB and controls sulfide production, the relative roles of the HNRB and chemolithotrophic NRB have not been established. In part, this is because the formulations of the media used for their enumeration have not been selective for these two individual types of NRB. That is, culture media used by different research groups often contained nutrients that allowed the growth of both HNRB and chemolithotrophic NRB. For example, the medium used by Davidova et al. (2001) contained thiosulfate, an electron donor for some chemolithotrophic NRB, and yeast extract, a potential electron donor for HNRB. Similarly, the medium used by Telang et al. (1999) for the enumeration of NR-SOB contained acetate, which would allow the growth of some HNRB.

Molecular biology techniques have also been used to monitor changes in oil field microbial communities. For example, two species of NR-SOB were isolated from an oil field water (Gevertz et al. 2000), and the DNA from these isolates has been used for reverse sample genome probing (RSGP) (Telang et al. 1997). The monitoring method is very specific, and without a DNA standard of a particular heterotrophic nitrate-reducing bacterium, the method is insensitive to the presence of that bacterium in oil field waters.

In Chapter 3, Eckford and Fedorak (2002b) used three different types of culture media to enumerate different nutritional types of NRB using most probable number (MPN) methods. The media used for the chemolithotrophic NRB were free of organic components to prevent the growth of HNRB, and the medium used for HNRB was free of reduced inorganic sulfur species to prevent the growth of chemolithotrophic NRB.

Because no previous study had specifically enumerated the different nutritional types of NRB in nitrate-amended sour oil field waters, this study used three media formulations (Chapter 3 and Eckford and Fedorak 2002b) to determine which types of planktonic NRB were stimulated in laboratory microcosms. Produced waters from three oil fields were used, and the chemical and bacterial changes in the microcosms were followed over time. In two cases, both the NR-SOB and HNRB increased in numbers and the sulfide was removed quickly. In the third case, only the NR-SOB increased in numbers and the sulfide was removed slowly.

4.2 Materials and methods

4.2.1 Produced waters for nitrate amendment

Produced water samples from three oil fields were collected in sterile, anaerobic serum bottles. Some of the characteristics of these souring oil fields and the samples are summarized in Table 4.1. Although oil fields P and N are from the same formation, they are from different pools that are about 5 km apart, and the fields are not connected. Further details on sampling and other oil field parameters were reported previously (Chapter 3 and Eckford and Fedorak 2002b). Oil field P was not being treated with biocides, but oil fields N and C were receiving biocides. The operators at oil field N turned off the biocides feed 1 week prior to sampling, to minimize the effects of biocides on this study, but the operators of oil field C did not stop the biocides feed during sample collection.

The samples were transported on ice to the laboratory and stored at 4°C until the serum-bottle microcosms were established to test the effects of nitrate amendment. Samples with elevated sulfide concentrations were chosen for the serum-bottle microcosm studies.

Table 4.1 Some characteristics of the three western Canadian oil fields that were sampled for this study

	P[a]	C	N
Nearest town	Stettler, Alberta	Coleville, Saskatchewan	Stettler, Alberta
Oil-bearing formation	Glauconitic	Bakken	Glauconitic[b]
Field depth (m)	1300	810	1400
Production started in	1994	1951	1992
Waterflooding started in	1994	1958	1994
Average water cut (%)	95	95	55
Sampling dates	Dec., 2000	July, 2001	May, 2001
Source of sample for nitrate amendment	Pre-injection site	FWKO[c]	Water storage tanks
Sulfide (mM)	0.78	2.7	0.94
$SO_4^=$ (mM)	5.9	0.56	4.4

[a] This sample was denoted "Pa3" in Chapter 3 by Eckford and Fedorak (2002b)
[b] Also referred to as the Upper Mannville formation (Davidova et al. 2001)
[c] Free Water Knock Out

4.2.2 Nitrate-amendment tests

The nitrate-amendment test was designed to observe the chemical and bacterial changes over 38 d following the addition of 10 mM NO_3^- to oil field waters. For each produced water studied, changes in the nitrate-amended samples were compared to changes in an unamended sample and a sterile control. The microcosms were established within 2 d of obtaining the produced water samples, so the MPN values determined on the original water samples were considered the time zero counts in the serum-bottle microcosms.

Serum bottles (158-mL) were flushed with O_2-free N_2, sealed and then sterilized by autoclaving. To each bottle, 100 mL of produced water sample was transferred aseptically and anaerobically from the sample collection bottles returned from the oil field. Care was taken to avoid adding oil to the microcosms so that the only source of electron donor was dissolved compounds present in the produced waters. No other potential electron donor was added to any of the microcosms, and all experiments were done without phosphate supplementation as was used by Wright et al. (1997) and Jenneman et al. (1997, 1999). McInerney et al. (1993) mention that most petroleum reservoir brines contain $SO_4^{=}$ but sources for nitrogen and phosphorus may be limiting. However, no special precautions were taken to use phosphate-free glassware, and the phosphorus content of the produced waters was not determined. The nitrate-amended and unamended microcosms were prepared in triplicate. For each test, two sterile controls were made from oil field water samples that had been autoclaved on two occasions for 30 min at 121°C.

A 1 M solution of KNO_3 was prepared using boiled distilled, deionized water. The solution was sparged with O_2-free N_2, sealed in a serum bottle, autoclaved and stored until needed. After the oil field waters were added to the serum bottles, 1 mL of the 1 M KNO_3 solution was added to the three serum bottles that were designated nitrate-amended. Immediately after the amended, unamended and sterile controls were prepared, time zero samples were removed for chemical analyses. The serum-bottle microcosms were incubated in the dark at room temperature (approximately 21°C), and at various times, samples were removed for chemical analyses and microbial counts.

Samples were removed from the serum-bottle microcosms every few days for chemical analyses until the sulfide was depleted; thereafter samples for chemical analyses were removed only when samples were taken for the MPN procedures. The MPN analyses were done repeatedly from one of the replicate serum-bottle microcosms (chosen at random). For oil field sample P, MPN determinations were done on samples taken from the microcosms at the time of inoculation and after 38 d of incubation. For oil field samples C and N, samples were withdrawn on days 0, 7, 14, 21, 28 and 38 for MPN determinations. Due to the large numbers of tubes of media required for the MPN method, the 3-tube MPN procedures were done on samples removed from the nitrate-amended and unamended microcosms, but only the 10^{-1} dilution from each sterile control was inoculated into the three different media. The sterile control was used to ensure sterility.

4.2.3 Microbial counting and analytical methods

Three-tube MPN procedures were done using HNRB medium for HNRB (Chapter 2 and Eckford and Fedorak 2002a), S8 medium for thiosulfate-oxidizing NRB (Chapter 2 and Eckford and Fedorak 2002a), modified CSB medium for NR-SOB (Chapter 3 and Eckford and Fedorak 2002b) and modified Butlin's medium for SRB (Chapter 3 and Eckford and Fedorak 2002b). The appropriate amount of NaCl was added to each medium to match the Cl^- concentration in each produced water sample (Chapter 3 and Eckford and Fedorak 2002b). Briefly, the HNRB medium contained nutrient broth as the electron donor. S8 medium contained thiosulfate as the electron donor. The modified CSB medium contained sulfide as the electron donor and was devoid of acetate. The modified Butlin's medium for SRB contained lactate as the electron donor. The inoculated tubes were incubated for 30 d at room temperature in the dark. The MPN tubes were scored positive for growth of (a) HNRB, if NO_3^- was consumed and N_2O was produced in the HNRB medium (Chapter 2 and Eckford and Fedorak 2002a; Fedorak et al. 2002); (b) thiosulfate-oxidizing NRB, if NO_3^- was consumed or NO_2^- formed in the S8 medium (Chapter 2 and Eckford and Fedorak 2002a); (c) SRB, if the iron nails in the medium turned black from the formation of FeS (Fedorak et al. 1987). Growth of NR-SOB in the modified CSB medium was scored by two methods based on color changes in the medium. This medium contained resazurin, a redox indicator, which was oxidized from colorless to pink by the formation of N_2O by the NRB (Jenneman et al. 1986b). Scoring the MPN tubes on the basis of the appearance of the pink color is referred to as method A. In some instances after 30 d of incubation, the medium in lower dilution MPN tubes was pink, and medium in some of the next higher dilutions was yellow. This was most evident in the enumerations of samples from oil field N. With extended incubation (up to 5 months), the medium in most of the tubes that were yellow turned pink. Scoring the MPN tubes positive on the basis of the appearance of either the pink or yellow color after 30 d incubation, is referred to as method B. The MPN results were compared by the statistical method of Cochran (1950).

With the exception of the first sample enumerated for NR-SOB (the time zero sample from oil field P), all of the inoculated tubes of modified CSB medium were incubated in an anaerobic chamber (Coy Laboratory Products Inc. Ann Arbor, MI) to ensure that O_2 from air would not cause the redox indicator to oxidize. NR-SOB, *Arcobacter* sp. strain FWKO B and *Thiomicrospira* sp. strain CVO (Gevertz et al. 2000), were obtained from Dr. G. Voordouw's laboratory (University of Calgary). These NR-SOB were used as positive controls for the modified CSB medium.

The oil field water samples and samples taken from the serum-bottle microcosms were analyzed for sulfide concentration using a kit purchased from CHEMetrics Inc. (Calverton, VA). An alkaline sodium nitroprusside spot test (Feigl and Anger 1972) was used to detect sulfide in the MPN cultures. Chloride, $SO_4^{=}$ and NO_3^- concentrations were determined by ion chromatography (Chapter 2 and Eckford and Fedorak 2002a) and NO_2^- was determined by a colorimetric method (Clesceri et al. 1998).

4.3 Results

Each of the produced water samples used for this study had elevated sulfide concentrations and $SO_4^{=}$, available for SRB (Table 4.1); conditions that are characteristic of souring oil fields. In addition, each sample contained HNRB, NR-SOB and SRB (Table 4.2). The maximum rate of NO_3^- consumption for each oil field shown in Table 4.2 was calculated using at least three time points. No thiosulfate-reducing NRB were detected in any of these samples. Work with the produced water P was done while the method for enumerating NR-SOB was first being implemented in this laboratory. After 30 d of incubation, some MPN cultures turned pink but scoring these tubes as positive did not yield utilizable MPN indices. Thus, the medium in each tube was tested for NO_2^-, and those tubes that contained NO_2^- were scored positive. The NO_2^- analyses were used as the basis for MPN results given for the initial NR-SOB MPN count in sample P in Tables 4.2

and 4.3. With additional experience enumerating NR-SOB in other samples, it was found that the MPN values based on the appearance of NO_2^- were essentially the same as those determined by method A (resazurin turning pink) (Chapter 3 and Eckford and Fedorak 2002b).

Table 4.2 Summary of bacterial numbers, nitrate-reduction rates and depletion of sulfide in nitrate-amended microcosms with produced waters from three different oil fields

Parameter	Oil field		
	P	C	N
Initial counts (MPN mL^{-1})			
HNRB	7.5	4.3x10^{2}	2.3 x10^{4}
NR-SOB	1.5x10^{3} [a]	2.1 x10^{5} [b,c]	9.3x10^{1} [b] 2.3 x10^{4} [c]
SRB	2.3x10^{3}	9.3 x10^{2}	2.3x10^{3}
Maximum counts (MPN mL^{-1})			
HNRB	4.3x10^{5}	9.3x10^{6}	4.3x10^{4}
NR-SOB	4.3x10^{6}	9.3x10^{7}	4.3x10^{4} [b] 2.3x10^{7} [c]
Maximum increase in			
HNRB MPN	57,000-fold	22,000-fold	0-fold [d]
NR-SOB MPN	2900-fold [a]	440-fold [b,c]	460-fold [b] 1000-fold [c]
Maximum nitrate-reduction rate (mM d^{-1})	0.68	1.4	0.4
Sulfide depleted by day	<4	3	27

[a] MPN value from NO_2^- analysis
[b] MPN value determined by method A
[c] MPN value determined by method B
[d] No statistical increase in MPN (*P<0.05*)

4.3.1 *Nitrate amendment in produced water from oil field P*

The sample used for nitrate-amendment studies was a "co-mingled" water from a pre-injection site. It was a mixture of produced waters and source water taken just before re-injection into the oil reservoir. The sample had a sulfide concentration of 0.78 mM (Table 4.1), and the initial number of NR-SOB was greater than the number of HNRB (Table 4.2). In the nitrate-amended microcosms, no sulfide was detected on day 4 (Figure 4.1a). Nitrate dropped from 10 mM at time zero to below detection by day 14 (Figure 4.1a), and the rate of NO_3^- loss taken from the linear portion of the NO_3^- loss curve was 0.68 mM d^{-1}. Nitrite was detected transiently in the nitrate-amendment microcosm (Figure 4.1a), indicating that nitrate reduction had occurred. The $SO_4^{=}$ concentration increased by 0.8 mM in the amended sample while the sulfide decreased by 0.78 mM, consistent with sulfide oxidation.

Table 4.3 Bacterial counts in the produced water sample from oil field P

	Counts (MPN mL^{-1})		
	Initial	After 38 d incubation	
Bacterial types	(MPN mL^{-1})	Nitrate-amended	Unamended
HNRB	7.5	4.3 $\times 10^5$	9.3 $\times 10^1$
NR-SOB	1.5$\times 10^3$ [a]	4.3 $\times 10^6$ [b]	3.9$\times 10^3$ [b]
SRB	2.3$\times 10^3$	2.3$\times 10^4$	2.3$\times 10^3$

[a] MPN value from NO_2^- analysis
[b] MPN value determined by method A

In contrast, the unamended microcosms showed a near stoichiometric reduction of $SO_4^{=}$ to sulfide with a loss of 5.8 mM $SO_4^{=}$ and gain of 5.6 mM sulfide (Figure 4.1b). Neither NO_3^- nor NO_2^- was detected in these unamended microcosms. The sterile control showed no change in $SO_4^{=}$ or sulfide concentrations, and no NO_3^- or NO_2^- was detected

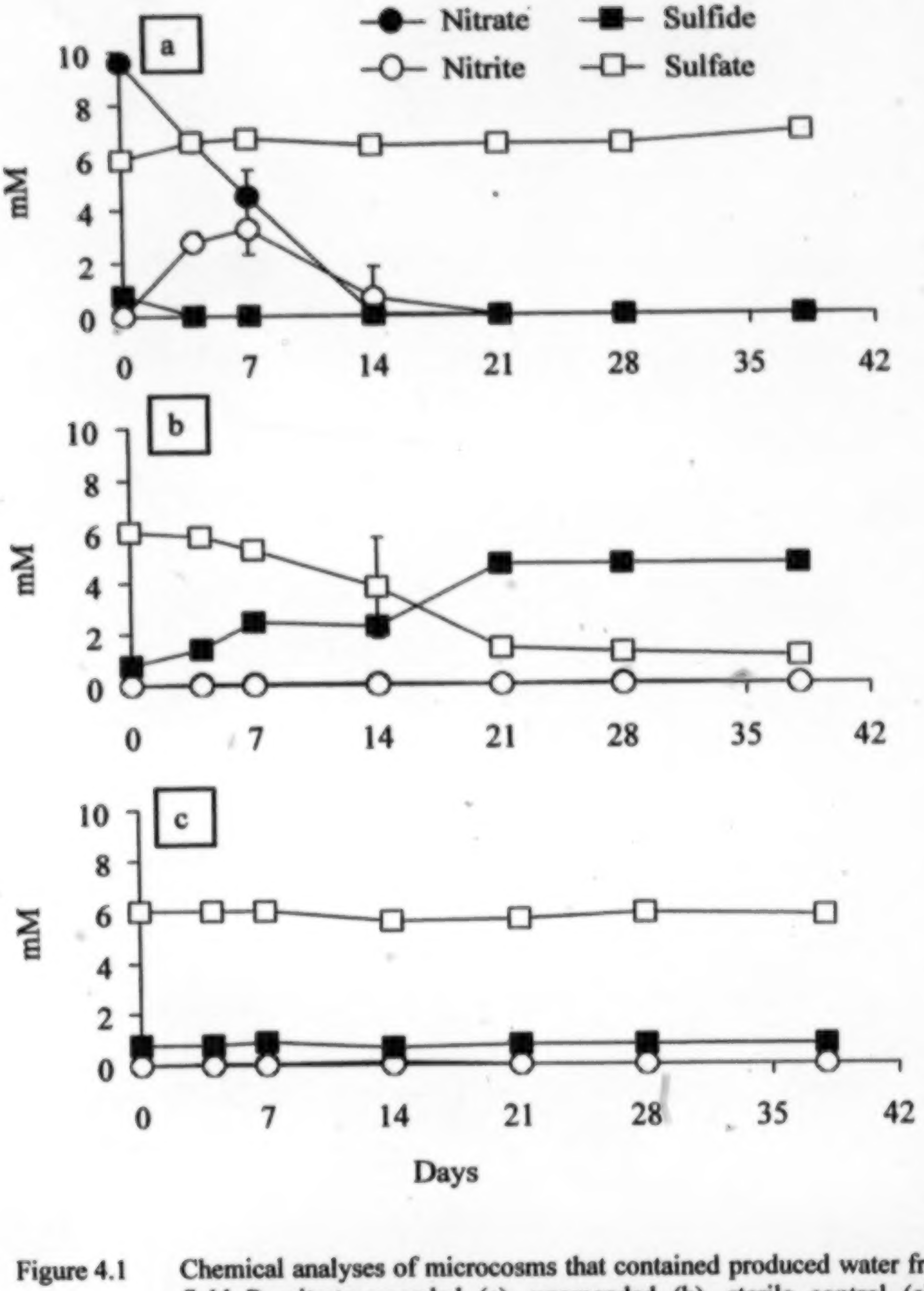

Figure 4.1 Chemical analyses of microcosms that contained produced water from oil field P; nitrate-amended (a), unamended (b), sterile control (c). The plotted values are means of three replicates. The error bars, which are often smaller than the size of the symbols, show one standard deviation

over the 38-d testing period (Figure 4.1c). The sterile controls for the other two oil fields showed the same pattern as Figure 4.1c, thus data from the other sterile controls are not presented. Samples taken from the sterile controls yielded no growth in any of the MPN tubes.

Data plotted in Figure 4.1 are the means obtained from triplicate microcosms, and in most cases, the error bars were smaller than the plotted symbol, indicating excellent reproducibility among the replicates. These data clearly show that nitrate amendment stopped sulfate reduction and contributed to the removal of the sulfide in the original produced water.

There were populations of HNRB and NR-SOB in this water (Table 4.3). On day 38, samples were taken from the nitrate-amended and the unamended microcosms. There was a marked increase in both NRB populations over the 38-d incubation (Table 4.3), with increases in HNRB and NR-SOB of about 57,000-fold and 2900-fold, respectively (Table 4.2). These increases were consistent with the depletion of NO_3^- from the microcosms (Figure 4.1a). The unamended microcosm showed a slight increase in the HNRB population ($P<0.05$) and no increase in the NR-SOB population ($P<0.05$) (Table 4.3). The SRB population showed a slight increase ($P<0.05$) in the amended microcosm and no change in the unamended sample in 38 d. Jack and Westlake (1995) also observed an increase in the number of SRB after 1.6 mM NO_3^- was added to a field test facility. In their study, the numbers of SRB increased 100-fold over 30 d.

4.3.2 Nitrate amendment in produced water from oil field C

A produced water sample from the FWKO at oil field C was chosen for this study because several other studies have described NR-SOB from these waters (Jenneman et al. 1997; Telang et al. 1997, 1999; Gevertz et al. 2000), and the oil field has a severe souring problem. The chemical analyses showed that there were some differences among the triplicate nitrate-amended microcosms over the incubation period. The results shown in Figure 4.2a are from the microcosm that was sampled for bacterial enumerations. Initially, this microcosm contained 2.7 mM sulfide which increased to 3.1 mM by day 1 and then dropped below detection by day 3 in the nitrate-amended microcosm (Figure

4.2a). The rate of NO_3^- consumption over the first 3 d was 1.4 mM d^{-1} (Table 4.2). The NO_3^- concentration then remained at about 5 to 6 mM for the rest of the incubation. The $SO_4^=$ concentration increased noticeably over the first 14 d of incubation, with a total increase of 3.5 mM by day 38, closely matching the 3.1 mM decrease in sulfide. The NO_2^- concentration was at a maximum of 1.8 mM on day 3 and then gradually decreased to 0.2 mM by day 38 (Figure 4.2a).

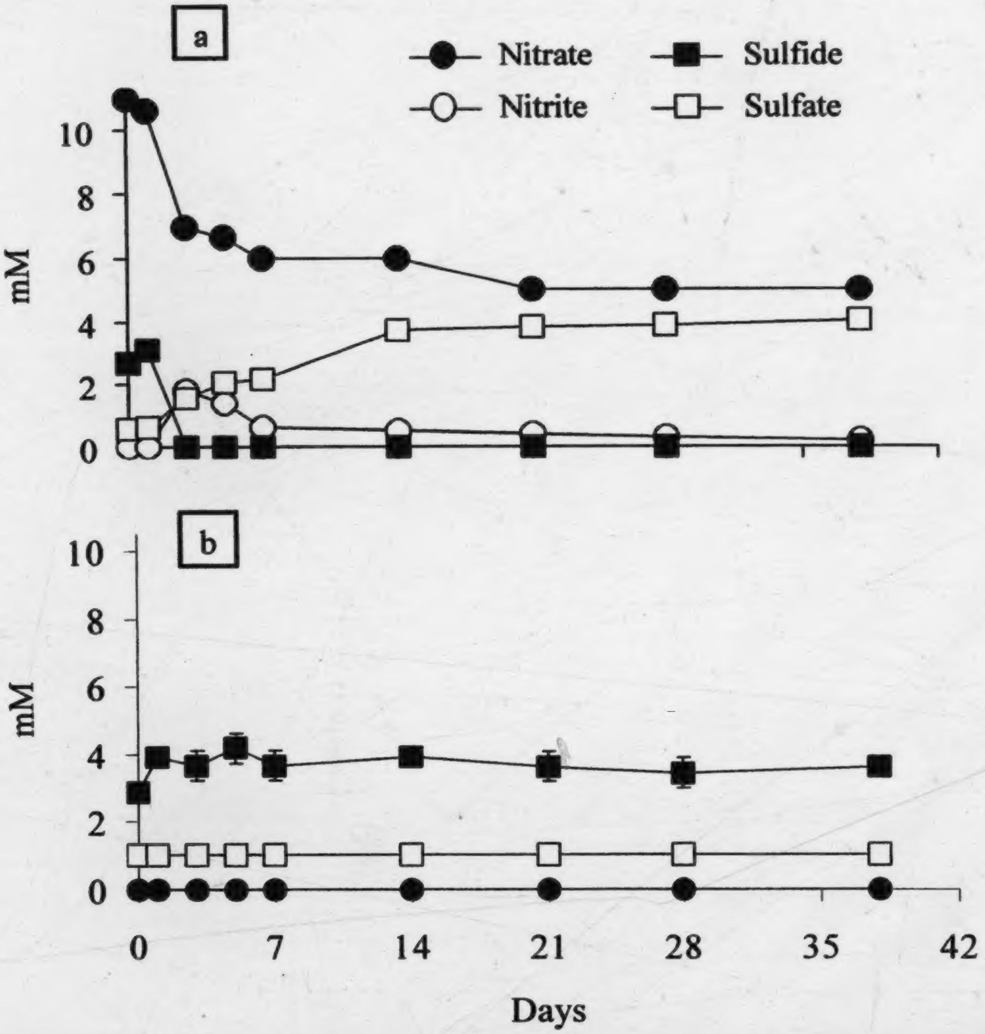

Figure 4.2 Chemical analyses of microcosms that contained produced water from oil field C; nitrate-amended (a), unamended (b). See text for discussion of the results of the other two replicate microcosms.

Chemical analyses of samples from the other two nitrate-amended microcosms that contained produced water from oil field C showed a slight increase in sulfide over the first few days of incubation, and then a complete loss of sulfide as was observed (Figure 4.2a). In one of these microcosms, the $SO_4^=$ concentration remained stable at 0.6 mM during the 38-d period and after a drop in NO_3^- concentration during the first 8 d of incubation, the NO_3^- concentration remained at 9 mM for the duration of the incubation. No NO_2^- was detected in this microcosm. In the third microcosm, there was a rapid decrease in NO_3^- over the first 3 d followed by a gradual decrease in NO_3^-. The final concentration of NO_3^- was 4.5 mM on day 38. This was accompanied by an increase in NO_2^- concentration to 4 mM by day 38. Sulfate accumulated in this microcosm. Figure 4.2a shows the final $SO_4^=$ concentration at 3 mM. The reasons for the discrepancies among these triplet microcosms is unknown, and these were the only three microcosms in the entire study that showed this high variability.

The sample from oil field C was the only sample collected while biocides were being injected into the produced water, and the presence of the biocides may have influenced the results. Nonetheless, all three nitrate-amended microcosms demonstrated NO_3^- consumption and sulfide removal. There was little variability among the three unamended microcosms, so chemical parameters in Figure 4.2b are the means of the triplicate microcosms. The sulfide increased to 4 mM by day 5, and the $SO_4^=$ remained fairly steady at from 0.68 mM to 0.54 mM throughout the testing period. Neither NO_3^- nor NO_2^- was detected in the microcosms.

Bacterial enumerations were done on samples from one nitrate-amended and one unamended microcosm at intervals of 7 to 10 d. The MPN results are shown in Figure 4.3. Initially, the number of NR-SOB (2.1×10^5 mL^{-1}) was much greater than the number of HNRB (4.3×10^2 mL^{-1}) (Table 4.2). There was no increase in the numbers of HNRB (Figure 4.3a) or NR-SOB (Figure 4.3b) in the unamended microcosm that contained produced water from oil field C. In contrast, there was a rapid increase in the numbers of HNRB and NR-SOB by day 7 in the nitrate-amended microcosm (Figures 4.3a and 4.3b). The numbers of HNRB and NR-SOB increased 22,000-fold and 440-fold, respectively. These proliferations occurred during the time when NO_3^- consumption was the most rapid and sulfide was depleted from the microcosm (Figure 4.2a). At day 7, the numbers of

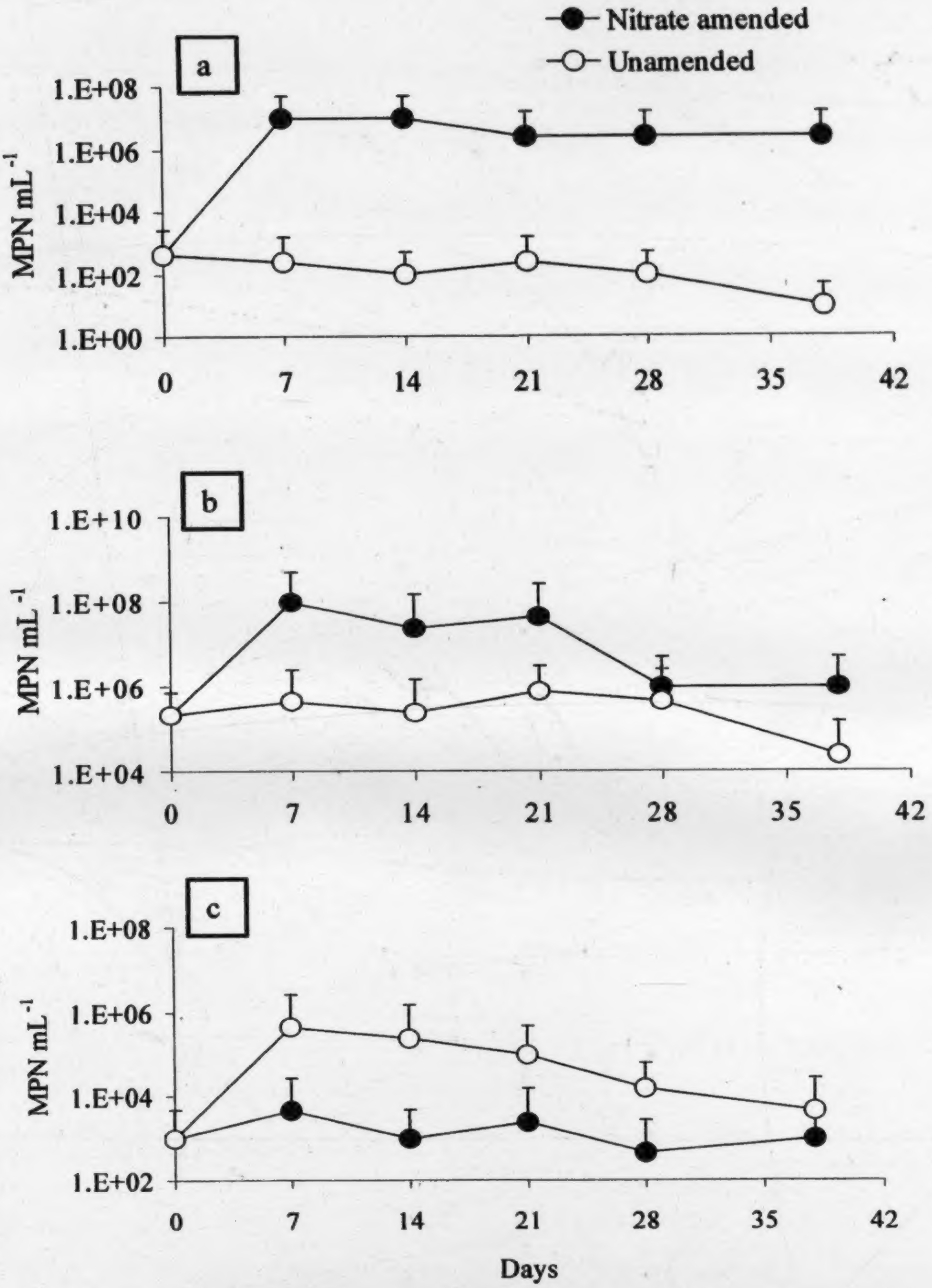

Figure 4.3 HNRB (a), NR-SOB (b) and SRB (c) counts in samples from a microcosm that contained produced water from oil field C. The NR-SOB MPN values were determined by method A, and the error bars show the 95% confidence interval of the MPN values.

HNRB and NR-SOB were 9.3×10^6 mL^{-1} and 9.3×10^7 mL^{-1}, respectively. Over the remainder of the incubation, the HNRB numbers remained high, whereas the NR-SOB numbers dropped to near their original count (Figures 4.3a and 4.3b, respectively). The SRB numbers did not change in the amended microcosm and showed a slight increase in the unamended microcosm, with a maximum at day 7 (Figure 4.3c).

4.3.3 Nitrate amendment in produced water from oil field N

The water sample for oil field N was taken from the outlet of the storage tanks, just before re-injection into the reservoir. The initial sulfide concentration in the microcosms was 0.94 mM (Table 4.1). Nitrate consumption was observed over the first 13 d (Figure 4.4a), and the utilization rate of NO_3^- was 0.4 mM d^{-1} (Table 4.2). Unlike the results from the other two oil field waters, sulfide persisted until after day 20, when it decreased to below detection on day 27 (Figure 4.4a). The $SO_4^=$ concentration remained stable between 4 and 5 mM, and NO_2^- was detected once (day 3).

In the unamended microcosms, there was a rapid decrease in $SO_4^=$ between days 3 and 9 (Figure 4.4b). This decrease in 4.4 mM $SO_4^=$ was accompanied by an increase of about 5 mM sulfide. Neither NO_3^- nor NO_2^- was detected in these unamended microcosms. The results in Figure 4.4 again illustrate that nitrate amendment controls sulfide formation.

As shown in Table 4.2, the produced water from oil field N initially contained 2.3×10^4 HNRB mL^{-1} and 93 NR-SOB mL^{-1} (based on enumeration method A). The increase in the NRB population in the nitrate-amended microcosms was much lower than was seen in the nitrate-amendment tests for oil fields P and C. The numbers of HNRB in the amended microcosms were the same ($P<0.05$) as those in the unamended microcosms (Figure 4.5a) until day 38. There was no statistically significant increase in the number of HNRB during this incubation (Table 4.2). The number of NR-SOB increased 460-fold ($P<0.05$, based on method A) during the first 7 d of the incubation (Figure 4.5b) and remained between 4.3×10^2 mL^{-1} and 4.3×10^4 mL^{-1} over the duration of the experiment. Although the numbers of NR-SOB in the nitrate-amended microcosm were also statistically ($P<0.05$) higher than those in the unamended microcosm on days 21 and 38, the MPN values were quite similar in the two sets of microcosms (Figure 4.5b). There was an increase in the SRB number during the first 7 d incubation for the unamended microcosm (Figure 4.5c), and there was a rapid consumption of $SO_4^=$ (Figure 4.4b). The SRB numbers did not increase in the amended microcosm (Figure 4.5c).

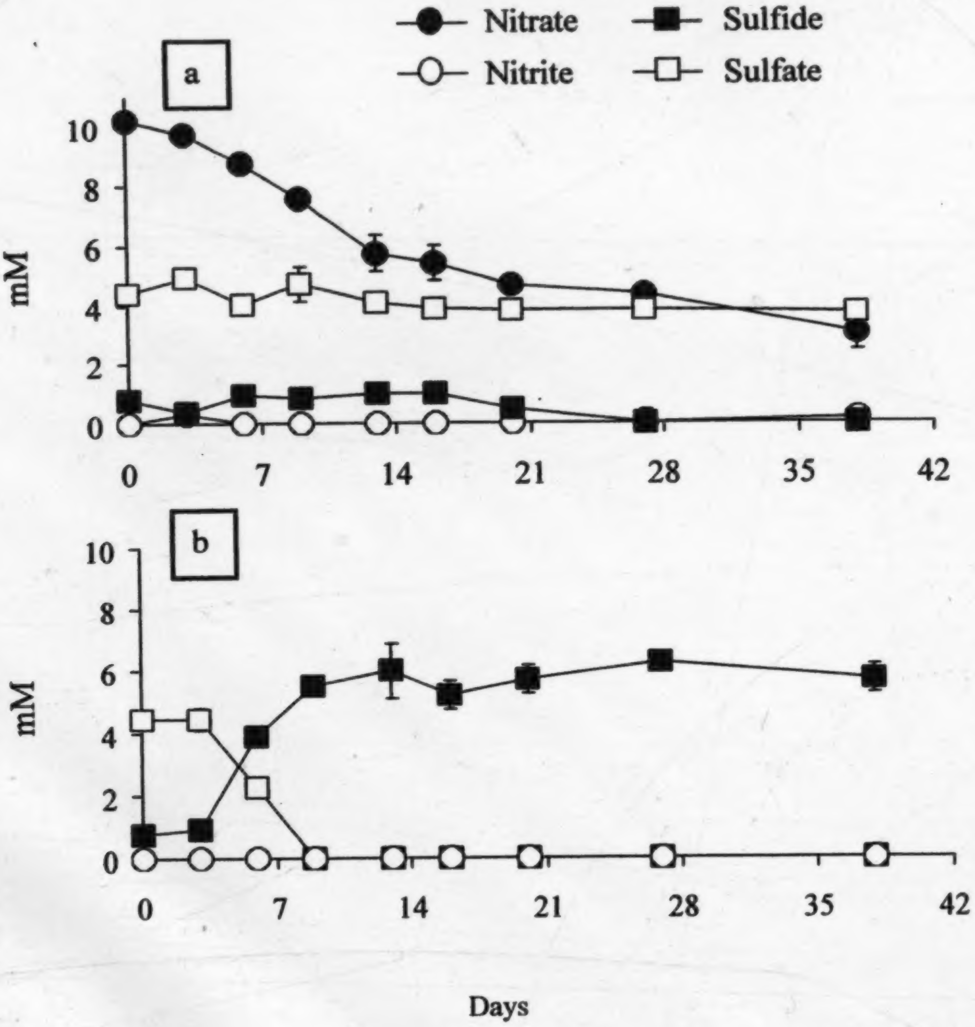

Figure 4.4 Chemical analyses of microcosms that contained produced water from oil field N; nitrate-amended (a), unamended (b). The plotted values are means of three replicates. The error bars, which are often smaller than the size of the symbols, show one standard deviation.

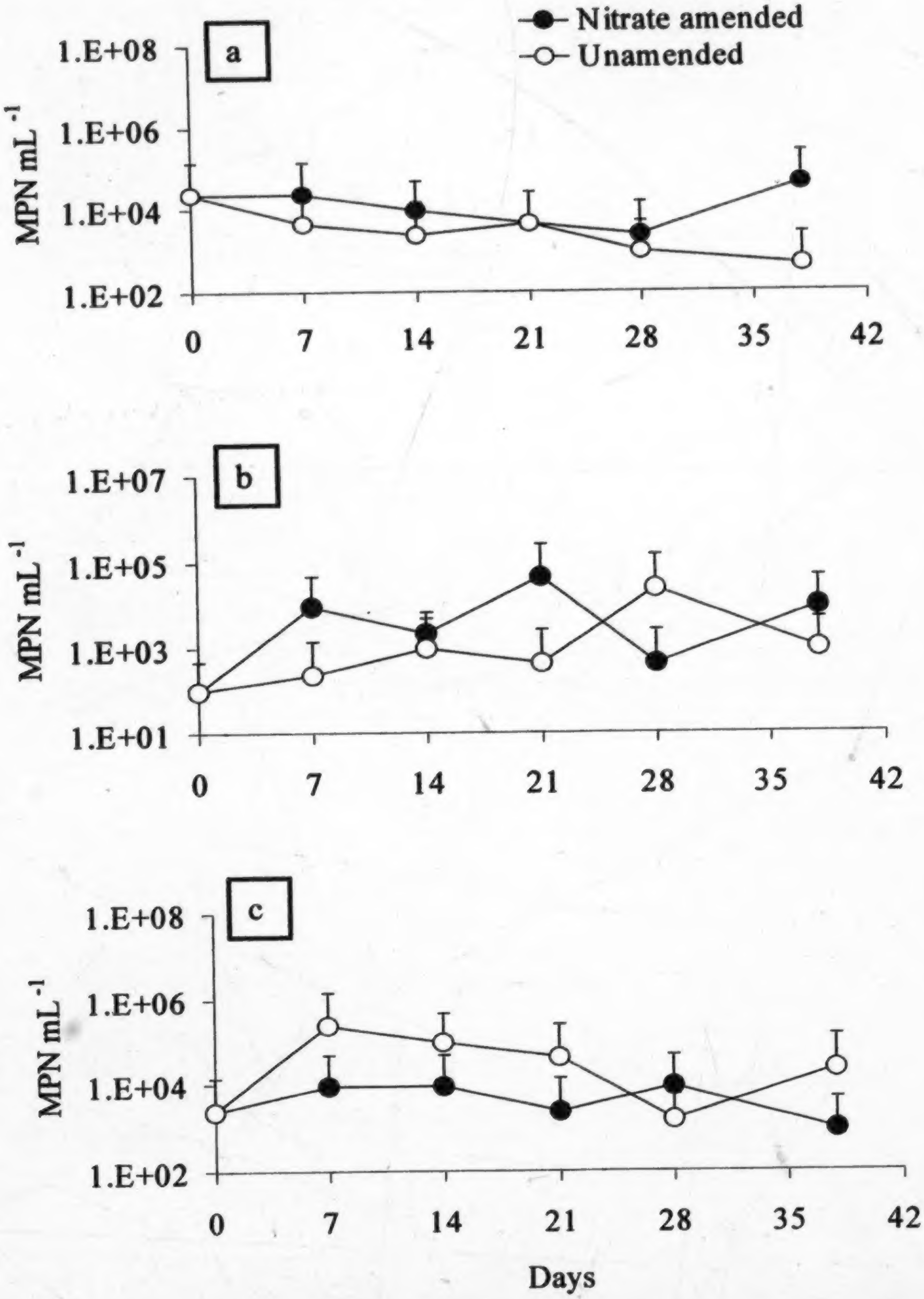

Figure 4.5 HNRB (a), NR-SOB (b) and SRB (c) counts in samples from a microcosm that contained produced water from oil field N. The NR-SOB MPN values were determined by method A, and the error bars show the 95% confidence interval of the MPN values.

4.3.4 Color changes in the serum bottles and the modified CSB medium

Generally, the liquid in the unamended microcosms was colorless or black and it did not change over the incubation period. A black precipitate, which was presumably iron sulfide, formed as a result of sulfide production by the SRB, was observed in the unamended microcosms at the end of the incubation period.

A transient yellow color appeared in the liquid of the nitrate-amended microcosms that contained oil field waters C or N. These liquids then became grey or brown as the sulfide was being removed. For example, the nitrate-amended microcosms that contained produced water from oil field N turned yellow at day 2, lost this color at day 3 and then turned yellow again at day 13, remaining yellow until day 20. The water in the microcosms then turned grey and remained grey until the end of the experiment. This transient yellow color has been observed by others who carried out studies with nitrate-amendment microcosms (Gevertz et al. 1995, Jenneman and Gevertz 2000), and the yellow color was attributed to the formation of polysulfides (Jenneman and Gevertz 2000).

As expected from the literature (Jenneman et al. 1996, Telang et al. 1999), many of the inoculated tubes of modified CSB medium turned pink as the resazurin was oxidized by N_2O, produced by the NR-SOB (Jenneman et al. 1986b, 1996; Telang et al. 1999). However, some of the culture medium turned yellow. For example, after 30 d incubation, many culture tubes of modified CSB medium inoculated with dilutions of the produced water from oil field N were pink. Several tubes of medium at the next higher 10-fold dilutions of the sample were yellow. These tubes were incubated longer to see if the cultures would turn pink. By 2 months, many of the MPN tubes that were yellow had turned pink. At 5 months, the majority of the tubes with yellow medium had turned pink. These cultures were then tested for sulfide consumption. The medium that turned from yellow to pink was devoid of sulfide. Similarly, no sulfide was detected in any of the tubes in which the medium turned pink within 30 d of incubation. In freshly prepared modified CSB medium, NO_3^- is abundant and sulfide (the electron donor) is the limiting nutrient (Chapter 3 and Eckford and Fedorak 2002b). Thus, the depletion of sulfide is indicative of the presence and growth of NR-SOB. These findings led to the use of method B for the calculation of the MPN values, which considered tubes to be positive for the growth of NR-SOB if the medium was pink or yellow after 30 d of incubation.

Figure 4.6 compares the NR-SOB MPN results obtained by applying methods A and B to samples from microcosms that contained produced water from oil field N.

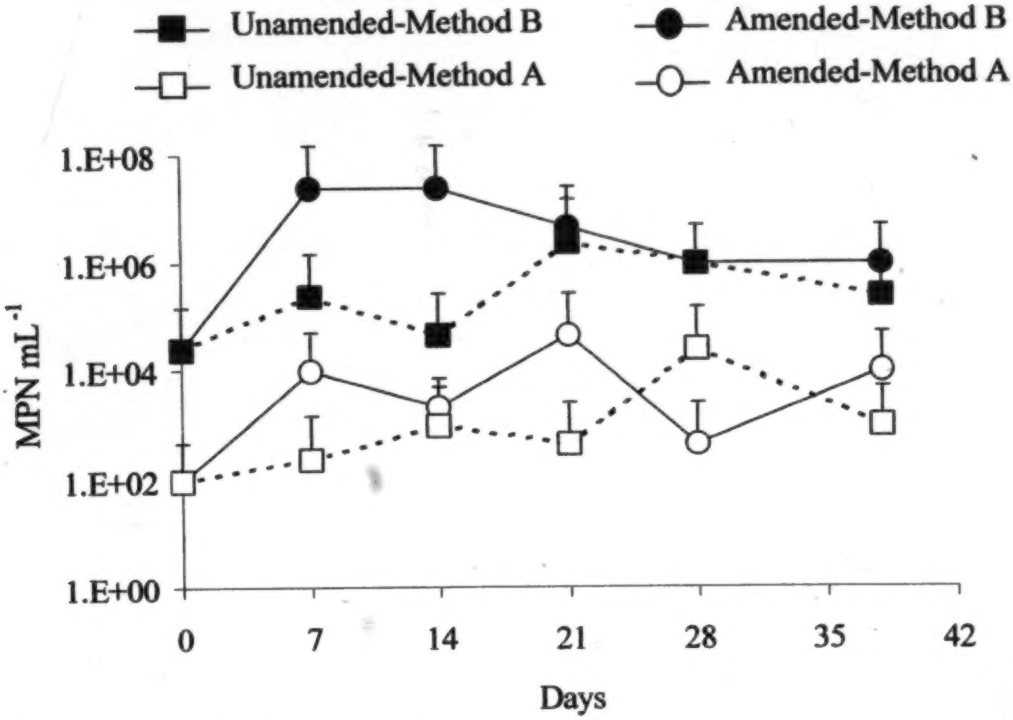

Figure 4.6 Comparison of NR-SOB MPN values determined by methods A and B, and the error bars show the 95% confidence interval of the MPN values. Samples were taken from microcosms that contained produced water from oil field N.

Data from the unamended and nitrate-amended serum bottles are included in Figure 4.6. The MPN values determined by method B were always higher than those determined by method A. In general, MPN results from method B gave values that were 100- to 10,000-times higher than MPN results from method A. Indeed, the time zero count, which was simply the NR-SOB counts in the produced water sample collected from oil field N, was 2.3×10^4 mL^{-1} by method B and only 93 mL^{-1} by method A.

Among the samples from the nitrate-amendment experiments with produced water from oil field C, only one tube of modified CSB turned yellow. From oil field P, only two tubes of medium turned yellow. For these two water samples, there was no significant difference (*$P<0.05$*) between the MPN values for the NR-SOB numbers determined by method A or B.

The MPN results determined by method B (Figure 4.6, solid symbols) showed that there was a rapid, 1000-fold increase in NR-SOB numbers during the first 7 d of incubation, and this elevated count remained high until after day 14. After day 14, the numbers dropped to the same levels as those in the unamended microcosm. The most rapid decrease in NO_3^- concentration also occurred during this 14-d period (Figure 4.4a).

4.4 Discussion

Any method used to study the composition of a microbial community has its limitations. Madsen (2000) has recently reviewed many of the major nucleic acid based methods used for characterizing naturally occurring microorganisms, and he has listed limitations for each method. Any method that relies on cultivation of microorganisms, such as the MPN methods used in this study, can never detect all of the microorganisms present in an environmental sample (Herbert 1990). Established media formulations were used, with little or no change to their compositions, for the enumerations in this study. The medium for SRB contained lactate as an electron donor. Thirty-nine of the 54 species (72%) of SRB listed by Stackebrandt et al. (1995) grow on lactate, which is used in the medium recommended by the American Petroleum Institute for the enumeration of SRB (McInerney and Sublette 2002). The S8 medium is recommended by the American Type Culture Collection (ATCC) for the growth of thiobacilli. The medium and method used to enumerate HNRB is a standard procedure used for soil analysis (Tiedje 1982). Only one-half of the concentration of nutrient broth in this medium was used to make it more suitable for water samples, and comparisons of counts obtained with the half-strength and full strength medium showed there were no differences in the MPN values (unpublished results). The CSB medium, used by others (Telang et al. 1999), was modified by omitting acetate. This modification made the medium more selective for the chemolithotrophic NR-SOB.

Previous research has investigated the presence of NRB and the use of NO_3^- to control sulfate reduction in produced waters from oil fields C (Jenneman et al. 1997, 1999; Telang et al. 1997; Gevertz et al. 2000) and N (Davidova et al. 2001; Chapter 3 and Eckford and Fedorak 2002b). Although the numbers of NRB in oil field P have been reported (Chapter 3 and Eckford and Fedorak 2002b), no previous studies on controlling sulfide production with nitrate amendment to produced waters from this oil field have been done.

Regardless of the source of the produced water, each microcosm was amended with 10 mM NO_3^- in this study. This was the concentration used by Davidova et al. (2001) in their studies with samples from oil field N. Various researchers have used different concentrations of NO_3^- to inhibit sulfate reduction in laboratory studies. For example, Londry and Suflita (1999) reported that 16 mM NO_3^- prevented sulfide accumulation in microcosms that contained oily sludge wastes. Five millimolar NO_3^- was sufficient to remove sulfide from produced waters from an oil field in Oklahoma (Davidova et al. 2001) and from oil field C (Gevertz et al. 1995). In their studies of four west Texas oil fields, Wright et al. (1997) amended serum-bottle cultures with 40 mM NO_3^- to stimulate sulfide removal. In this study, amendment with 10 mM NO_3^- was sufficient to control sulfate reduction and remove existing sulfide from each of the three produced waters (Figures 4.1, 4.2 and 4.4). Only microcosms with produced water from oil field P (Figure 4.1a) consumed all of the NO_3^- over the duration of the incubations. After 38 d incubation, there was 4 to 9 mM NO_3^- in the serum bottles that contained produced water from oil field C and 3 to 4 mM in the microcosms that contained produced water from oil field N. These results show that there are differences in the amount of NO_3^- consumed by planktonic microorganisms in waters from different oil fields. The differences could be due to the types of NRB that may be present in the oil fields or to the waters themselves. The waters from all the oil fields were treated with biocides and corrosion inhibitors (personal communication from oil field workers). The biocides were not being used at the time of water collection for oil field P, had been turned off 1 week before sample collection for oil field N and were being used at the time of water collection for oil field C. Oil fields are all very unique environments and can become complicated when chemicals are added to improve oil recovery (McInerney and Sublette 2002). These complications could influence the amount of NO_3^- that is used by NRB during nitrate amendment to oil fields.

The actual NO_3^- concentration required in the water handling facilities at an oil field may be higher if biofilms have formed in the pipes and storage tanks. Typically, higher concentrations of biocides are required to control microbial activities in biofilms relative to planktonic microorganisms (Ruseska et al. 1982) and the same is likely true for NO_3^- treatment. However, Reinsel et al (1996) found that lower concentrations of NO_3^- were required to maintain inhibition of SRB activity after the initial NO_3^- was added.

Some investigators have supplemented oil field waters with both NO_3^- and phosphate to stimulate microbial sulfide control (Telang et al. 1997, Wright et al. 1997). Only NO_3^- was added in the current study to decrease the cost of amendment in the oil field. The results indicated that sulfide removal occurred in all three produced waters without added phosphate.

Laboratory studies with *Thiobacillus denitrificans* strain F, a sulfide tolerant strain of NRB, showed that its growth could control biogenic sulfide production (Sublette et al. 1994, McInerney et al. 1996). Thus, oil field waters were screened for this group of chemolithotrophic NRB but none was detected in any of the samples taken from the microcosms. The S8 medium used in the studies supported the growth of strain F (Chapter 3 and Eckford and Fedorak 2002b) and *T. denitrificans* ATCC 23642 (Chapter 2 and Eckford and Fedorak 2002a). These results indicate that the three oil field waters did not contain nitrate-reducing thiobacilli that contributed to sulfide removal.

Several studies have estimated the number of NR-SOB in oil field waters using a MPN method which relies on the oxidation of resazurin by microbially produced N_2O. This turns the medium pink which is then scored positive for growth of NR-SOB. Oil field C has been studied extensively by this method (Gevertz et al. 1995; Jenneman et al. 1996, 1997; Telang et al. 1999), and Telang et al. (1999) enumerated NR-SOB in five additional oil fields using this method. None of the previous publications have reported the MPN medium, used during enumerations, as producing the transient yellow color observed with samples of oil field N produced water, although Telang et al. (1999) mentioned, while using acetate-containing CSB medium during enrichment work, that "enrichment of west Texas brines in CSB media led to development of a yellow colour, indicating formation of polysulfide", and "the resazurin indicator did not turn pink, suggesting that sulfide oxidation was incomplete".

For this study, after the yellow medium turned pink, consumption of sulfide was verified, indicative of the presence of NR-SOB. The sulfide analysis justified the use of method B for enumerating the MPN tubes. The Appendix examines abiotic removal of sulfide by NO_2^- in mineral medium. In an experiment, in 14 d, media with 5 and 10 mM NO_2^- turned pink and were depleted of sulfide, and media with 2, 1 and 0.5 mM NO_2^- turned yellow and sulfide remained (Figures A.1a and A.3). These results indicate that it is possible that NO_2^-, produced by NR-SOB from oil field N, may have removed the sulfide in the modified CSB medium over a 5-month period.

The fact that samples of the produced water from oil field N behaved very differently in the modified CSB medium suggests that a different type of NR-SOB exists in this oil field produced water. The NR-SOB in oil field C appear to produce N_2O (which oxidizes the resazurin) much more quickly than the NR-SOB from oil field N. The two NR-SOB that have been described in detail, strains CVO and FWKO B (Gevertz et al. 2000), were isolated from oil field C, and they do not produce the transient yellow color in the modified CSB medium.

Loka Bharathi et al. (1997) isolated over 100 strains of anaerobic colorless NR-SOB from seawater and a sulfide-rich creek. They presented data showing that different isolates oxidized sulfide at different rates. For example, one isolate oxidized all of the sulfide in the medium within 9 d, whereas another isolate oxidized only 2.9% of the sulfide in the same time. Thus, it is possible that the NR-SOB in the produced water from oil field N oxidize sulfide at a slower rate than the NR-SOB from oil field C. The slower rate of sulfide oxidation would yield a slower rate of nitrate reduction to N_2O. Consequently, the NR-SOB from oil field N would take a longer time to turn the MPN medium pink than those NR-SOB from oil field C. Indeed, this is what was observed.

The different response of the NR-SOB from oil field N in the modified CSB medium suggests that these bacteria grow more slowly than those from oil field C. This slower growth may have contributed to the low maximum nitrate-reduction rate in the microcosms that contained water from oil field N (Table 4.2) and the slower depletion of sulfide from these microcosms (Table 4.2). The NR-SOB from oil field N may be more sensitive to soluble organics that are known to sometimes inhibit chemolithotrophs, or they might be inhibited by sulfide concentrations in the produced water and modified CSB medium.

Very few other oil fields have been studied for the presence of NR-SOB. Using molecular biology techniques, Voordouw et al. (1996) detected sulfide oxidizers in several oil field waters from western Canada, but the growth characteristics of these bacteria were not determined. Using a MPN method with acetate-containing CSB medium, Telang et al. (1999) detected NRB in five of the six oil fields that they sampled.

This is the first investigation which specifically monitored the changes in numbers of HNRB and NR-SOB in nitrate-amended oil field waters. Some studies have monitored changes in the NR-SOB population size by RSGP (Telang et al. 1997, 1999; Nemati et al. 2001), whereas other studies have used culture methods with a medium that was not selective for a particular nutritional type of NRB because it contained reduced sulfur species along with organic compounds from filter-sterilized oil field brine (Gevertz et al. 1995, Jenneman et al. 1997), yeast extract (Davidova et al. 2001) or acetate (Telang et al. 1999). Many simple hydrocarbons, such as benzene, toluene, ethylbenzene, *m*-xylene, naphthalene and C_6 to C_{12} alkanes that would dissolve in produced waters, can be degraded by HNRB (Burland and Edwards 1999, Widdel and Rabus 2001). Acetate and other short chain fatty acids are common in oil field waters (Barth 1991, Magot et al. 2000), and these compounds are known to serve as a substrate for heterotrophic nitrate reduction (Beauchamp et al. 1989). The modified formulation of CSB medium lacked acetate and contained only inorganic compounds to select for chemolithotrophic NR-SOB.

Bacterial counts, after NO_3^- addition, were done only twice on samples from the microcosms that contained produced water from oil field P (Table 4.3). These showed large increases in numbers of HNRB (57,000-fold) and NR-SOB (2900-fold) after 38 d incubation (Table 4.2). To gain a better understanding of the community dynamics, bacterial counts were done on six occasions over the 38-d incubations of microcosms with waters from the other two oil fields (Figures 4.3 and 4.5). The initial MPN of NR-SOB in produced water from oil field C was 2.1×10^5 mL^{-1} (Table 4.2), determined using modified CSB medium. Previously, the numbers of "NR-SOB" have been determined with medium containing filter-sterilized brine from this oil field or acetate. Using filtered brine, the values were about 10^4 to 10^5 mL^{-1} at injection wells and <10 mL^{-1} at oil-producing wells (Jenneman et al. 1997). Using acetate-containing medium, the number of "NR-SOB" was reported to be 10^6 mL^{-1} (Telang et al. 1999). The counts in this study were about the same for filter-sterilized brine and lower than was observed with the acetate-containing CSB medium.

Figures 4.3a and 4.3b show that the increases in NRB numbers for oil field C occurred during the first 7 d of incubation. The number of HNRB increased 22,000-fold, and the increase in NR-SOB was only 440-fold (Table 4.2). A portion of the reservoir in oil field C was experimentally amended with NO_3^- in 1996 (Jenneman et al. 1997, 1999), and the numbers of NRB enumerated in filter-sterilized brine often exceeded 10^8 mL^{-1} during nitrate amendment (Jenneman et al. 1997). In the present study, the highest count of NR-SOB observed in the nitrate-amended microcosm from this oil field was 9.3×10^7 mL^{-1} (Table 4.2), in reasonable agreement with the observed field data after nitrate amendment.

Telang et al. (1997) collected produced water samples from oil field C before and after nitrate amendment to follow the changes in the microbial community by RSGP. Their master filter had DNA from 47 bacterial isolates, including the NR-SOB strain CVO, 26 different SRB and three heterotrophic bacteria that reduced NO_3^- to NO_2^-. By subjecting the total DNA extracted from the produced water to RSGP, they concluded that isolate CVO became the dominant community member immediately after nitrate injection and that no significant enhancement of other community members, including SRB, was observed (Telang et al. 1997). In this study, the data from the nitrate-amended microcosm that contained produced water from oil field C also showed an increase in the number of NR-SOB (from 2.1×10^5 mL^{-1} to 9.3×10^7 mL^{-1}) during the first 7 d of incubation (Figure 4.3b) and no increase in the numbers of SRB (Figure 4.3c). This is in agreement with the Telang et al. (1997) study.

Telang et al. (1997) also stated that, based on the RSGP analysis, none of the three heterotrophic isolates that produced NO_2^- from NO_3^- showed a strong increase as a result of nitrate amendment. They wrote, "It thus appears that of the community members represented on the filter, CVO is the primary benefactor from NO_3^- addition." Similarly, no increase in HNRB was reported in nitrate-amended serum-bottle experiments using produced water from oil field C and the same RSGP analysis (Nemati et al. 2001). In contrast, the present study results showed that the number of HNRB increased sharply during the first 7 d of incubation (from $4.3x10^2$ mL^{-1} to $9.3x10^6$ mL^{-1}) as shown in Figure 4.3a. Although the number of HNRB on day 7 was only one-tenth that of the number of NR-SOB, nitrate amendment caused a larger increase in the number of HNRB (a 22,000-fold increase) than the NR-SOB (a 440-fold increase shown in Table 4.2). Interestingly, the numbers of HNRB remained high over the duration of the incubation (Figure 4.3a), whereas the numbers of NR-SOB decreased after 21 d (Figure 4.3b).

The RSGP method is limited by the types of DNA standards that are spotted onto the master filter. For example, if the DNA from the HNRB that proliferated in the microcosms did not hybridize with the DNA of the three "standard" heterotrophic nitrate reducers, then the increase in population of HNRB could not be detected by the RSGP method. The MPN method that was used is a more general approach, which detects any HNRB that are culturable in the HNRB medium under the incubation conditions that were used in this study. The results demonstrated that the HNRB also benefited from NO_3^- addition to the water from oil field C.

The ability of nitrate amendment to stimulate and increase the numbers of NRB in microcosms that contained produced water from oil field N was demonstrated by Davidova et al. (2001). However, the MPN medium that they used was different from the media used in this study, and it is not clear which type of NRB proliferated in that study. The results showed a 570-fold increase in NRB number after 42 d of incubation (Davidova et al. 2001). In that study, there was no increase in the number of SRB in the nitrate-amended microcosms, consistent with the results presented in Figure 4.5c.

Sulfide was present until between days 20 and 27 in the nitrate-amended microcosms that contained produced water from oil field N (Figure 4.4a), in sharp contrast to the 3 to 4 d in the microcosms that contained samples from oil fields P and C

(Table 4.2). Davidova et al. (2001) observed active nitrate reduction in nitrate-amended microcosms that contained produced water from oil field N, and sulfide persisted at near 0.3 mM in these microcosms for 9 weeks. Similarly, an incubation time of nearly 14 weeks was required before the sulfide concentration in nitrate-amended microcosms that contained produced water from an oil field in Oklahoma decreased from approximately 4 mM to below detection limit (Davidova et al. 2001). Thus, although the activities of NRB control the net production of sulfide in produced waters, nitrate reduction does not always result in rapid sulfide consumption (for example, see Figure 4.4).

Voordouw et al. (1996) presented a model for an anaerobic sulfur cycle that might exist in an oil field. The cycle may be driven by the diffusion or convection of NO_3^- from surface layers into the reservoir. Nitrate provides an electron acceptor for the NR-SOB that oxidize sulfide to $SO_4^=$. This $SO_4^=$ serves as an electron acceptor for SRB that use H_2, organic acids or hydrocarbons as electron donors to reduce the $SO_4^=$ back to sulfide, thereby completing the sulfur cycle (Voordouw et al. 1996).

Assuming that the media formulations that were used would grow and distinguish different types of NRB, the results presented in Figure 4.4a appear to support the notion of anaerobic sulfur cycling in these microcosms that contained an abundant supply of NO_3^-. For the first 20 d, the concentrations of $SO_4^=$ and sulfide in the water from oil field N remained nearly constant, while there was a decrease in NO_3^- concentration. These observations suggest that NR-SOB consumed NO_3^- and produced $SO_4^=$, which was reduced back to sulfide by the SRB. Figure 4.6 shows that the number of NR-SOB increased substantially during the first week of incubation, presumably because of the added source of NO_3^-. In addition, there was no increase in the number of HNRB (Figure 4.5a), suggesting that they were not responsible for the decrease in NO_3^- concentration. Furthermore, the produced water in these microcosms turned yellow on day 2, lost this color on day 3, became yellow again on day 13 and remained yellow for 7 d thereafter. Others have attributed the yellow color in nitrate-amended microcosms to polysulfides (Jenneman and Gevertz 2000). The transient presence of polysulfides would indicate that transformations of inorganic forms of sulfur occurred, while the $SO_4^=$ and sulfide concentrations remained essentially constant, consistent with sulfur cycling. Although it was proposed that the SRB were active in these nitrate-amended microcosms, no increase in their numbers was observed (Figure 4.5c). However, it is possible that there were increases in the numbers of SRB and HNRB in the nitrate-amended microcosms, but the MPN media used for their enumeration may not have had the correct formulation to detect these types of NRB and SRB.

For the above scenario on anaerobic sulfur cycling to be feasible, the predominant electron donor used by the SRB, in the produced water from oil field N, could not serve as an electron donor for the HNRB. If a common electron donor was used, the HNRB would have a thermodynamic advantage (e.g. equations 4.2 and 4.3), and the HNRB would out-compete the SRB for that electron donor. The HNRB could also have a kinetic advantage and out-compete the SRB for an electron donor. The kinetic advantage of HNRB was shown in a study by Chidthaisong and Conrad (2000) using anoxic rice field soil and [2-^{14}C]acetate with 10 mM NO_3^-, 50 mM $Fe(OH)_3$ and 6.25 mM $SO_4^=$ added as electron acceptors. Results showed that there was competition for the carbon sources glucose and acetate when the different electron acceptors were used by various types of bacteria in the soil. The authors reported that "uptake of acetate was faster in the presence of either nitrate, ferrihydrite or sulfate" than in the control study with no addition of electron acceptors. The uptake rate constant for [2-^{14}C]acetate in the soil was reported as 6.84 h^{-1} for NO_3^- and 1.26 h^{-1} for $SO_4^=$. These results imply that HNRB consume acetate faster than SRB, thus they have a kinetic advantage as well as a thermodynamic advantage.

For the study described in this chapter, when the electron donor used by the SRB became depleted in the batch cultures, sulfate reduction would stop, but sulfide oxidation would continue because NO_3^- and sulfide were still available. This would lead to the depletion of the sulfide. The results in Figure 4.4a suggest that the electron donor for the SRB was all consumed after about 20 d incubation because sulfide was not detected on day 27. The nature of the electron donor in these microcosms is unknown. Field study results have shown that the depletion of the electron donor could occur. At the North Sea, Veslefrikk oil field, Thorstenson et al. (2002) reported "the fact that NRB dominated the bacterial community and the number of SRB dropped dramatically, suggests that competition for carbon may be a major contributor to the inhibition of SRB. This argument is only valid in a carbon limited system, which is the case for Veslefrikk, where oxygen is removed in a vacuum deaerator and no organic water injection additives are used". These results could indicate that organic additives may be needed for some oil fields.

The numbers of NR-SOB were stimulated by 440-fold to 2900-fold in the nitrate-amended microcosms that contained the three different oil field samples used in this study (Table 4.2). Nitrate addition to waters from oil fields P and C caused a much larger increase in the numbers of HNRB, which were stimulated 57,000-fold and 22,000-fold, respectively (Table 4.2). These gave maximum nitrate-reduction rates of 0.68 and 1.4 mM d^{-1}, respectively. The only microcosm in which there was no increase in HNRB was that which contained produced water from oil field N (Table 4.2, Figure 4.5a). In addition, this sample gave the lowest maximum nitrate-reduction rate of 0.4 mM d^{-1} (Table 4.2) and the longest time before sulfide depletion (approximately 27 d). These observations suggest that the activities of HNRB may play a key role in the overall removal of sulfide from produced waters, and that when only the NR-SOB are stimulated, sulfide persists for a longer period of time. The sulfide persistence was observed with produced water from oil field N. These occurrences may be related to the presence of electron donors that can be used by both the HNRB and the SRB. Under this condition, the HNRB would out-compete the SRB for the electron donor (based on thermodynamic considerations as shown in equations 4.2 and 4.3 or possibly kinetic considerations), thereby stopping sulfate reduction. This would terminate the anaerobic sulfur cycle because the sulfide that was oxidized to $SO_4^{=}$ by the NR-SOB would not be reduced back to sulfide by the SRB. Thus, the sulfide would be quickly depleted from the produced waters as observed in Figures 4.1a and 4.2a. In their survey of produced waters from oil field C, Gevertz et al. (1995) observed sulfide removal within 2 d in five of the six sample locations studied. A sample was not taken from the microcosms on day 2, but the sulfide was depleted from the produced water from oil field C by day 3 (Figure 4.2a). Thus, the data agree with those of Gevertz et al. (1995).

Without specifically enumerating HNRB, Wright et al. (1997) showed the importance of heterotrophic activity in sulfide removal. They studied waters from four west Texas oil fields to determine which amendments were required to stimulate sulfide removal. In two of the samples, addition of NO_3^- and phosphate were not sufficient to promote biological removal of sulfide over a 28-d incubation. However, sulfide removal was observed when acetate or formate plus vitamins or yeast extract were added to two of these waters that had been supplemented with NO_3^- and phosphate. Hitzman and Sperl (1994) observed that acetate and propionate were used by the heterotrophic denitrifying populations in several oil field waters, and this activity prevented the growth of SRB. They reported that the heterotrophic denitrifiers became the dominant group in the microbial community after nitrate amendment. Unfortunately, the description of the enumeration method was not adequate to evaluate the validity of their results.

Many recent studies on controlling sulfide production have focussed on NR-SOB (Jenneman et al. 1997; Telang et al. 1997, 1999; Gevertz et al. 2000). Most of the emphasis has been on the strictly anaerobic *Arcobacter* sp. strain FWKO B and *Thiomicrospira* sp. strain CVO. Both of these strains are obligate chemolithotrophs (Gevertz et al. 2000), so they would not grow in the medium that was used for enumerating HNRB, which was devoid of sulfide. Although there appear to be no reports of oil field waters containing NR-SOB that can grow heterotrophically, Robertson and Kuenen (1983) described a bacterium with this capability. The bacterium, now known as *Paracoccus pantotrophus* (Rainey et al. 1999) [formerly *Paracoccus denitrificans* (Ludwig et al. 1993) and *Thiosphaera pantotropha* strain GB17 (Robertson and Kuenen 1983)], was isolated from a denitrifying effluent treatment system. It is a facultative anaerobe and facultative chemolithotroph that uses NO_3^- as an electron acceptor. It grows chemolithotrophically with sulfide as an electron donor or heterotrophically with a variety of organic compounds (including acetate, lactate, glucose) and casamino acids (an acid digest of casein, treated to eliminate or reduce vitamins) as electron donors (Robertson and Kuenen 1983).

The presence of NR-SOB that are facultative chemolithotrophs (like *P. pantotrophus*) in the microcosms would confound the assessment of the roles of the strictly chemolithotrophic NR-SOB and the HNRB in controlling sulfide in the produced waters. This is because a facultative autotroph would likely grow in both modified CSB medium used to enumerate the chemolithotrophic NRB and in medium used to enumerate HNRB. Although the methods used in this study would not clearly indicate the presence of facultatively chemolithotrophic NRB, the results suggest that these microorganisms were not abundant in the microcosms. That is, if facultatively chemolithotrophic NRB constituted the dominant portion of the community, then nitrate amendment would markedly increase their numbers, and this increase would be reflected to essentially the same extent in both the modified CSB medium and the medium for HNRB. However, the data in Table 4.2 do not show this trend. For example, in the samples from oil field P, the maximum increase in the MPN values for the HNRB was 57,000-fold and that for the NR-SOB was only 2900-fold. The data from oil field C show an even greater difference between the increases in the MPN values, with that of the HNRB increasing by 22,000-fold and that of the NR-SOB increasing only 440-fold. The data from oil field N (Table 4.2) present a different situation in that there was no increase in the HNRB numbers, while there was a 1000-fold increase in the NR-SOB numbers. Clearly, none of these sets of data shows a similar increase in the numbers of HNRB and NR-SOB, suggesting that facultatively chemolithotrophic NRB are not the major type of NRB in these produced waters. However, specific studies are required to better assess whether facultatively chemolithotrophic NRB play a role in controlling sulfide concentrations in oil field waters.

The objective of this research was to use different media to determine which types of planktonic NRB were stimulated in laboratory microcosms containing produced waters amended with NO_3^-. This was the first study to specifically monitor the numbers of HNRB in this type of experimental system. It was thought that their numbers would increase with time, but the results presented in Table 4.2 were somewhat surprising. That is, in oil fields P and C, the stimulation of the HNRB far exceeded that of the chemolithotrophic NR-SOB, whereas in oil field N the converse was true. In addition, the findings strengthen the notion that the activities of the HNRB may help control sulfide removal by interrupting the anaerobic sulfur cycle. In retrospect, determining the types and quantities of the organic compounds dissolved in the produced waters would have provided valuable information to help assess which electron donors were key to the anaerobic processes that took place after nitrate amendment. However, this was not done in this study. Now that the importance of the HNRB has been demonstrated, characterization of the dissolved organic electron donors should be addressed in subsequent studies.

In their review, Magot et al. (2000), mentioned that the organic acids formate, propionate, butyrate and benzoate are commonly detected in oil reservoirs, and acetate is the most abundant organic acid. They mention that organic acids are often but not always present in oil reservoirs. Concentrations of organic acids can be more than 20 mM. Naphthenic acids can be present at concentrations up to 100 mM. Barth (1991) reported the organic acids that were found in oil reservoirs on the Norwegian continental shelf. Acetic acid was the major organic acid detected in 21 formation water samples followed by propanoic acid. Other acids that were found in the same water samples were butanoic, pentanoic, hexanoic, benzoic and formic. The organic acids were typically weak acids that exist as anions in formation water with pH >5. Reinsel et al. (1996) measured organic acids in the Kuparuk, Alaska oil field and found 10.3 mM acetate, 1.0 mM propionate, 0.08 mM *n*-butyrate, 0.01 mM isobutyrate and no formate.

In summary, this study showed that the MPN technique used for enumerating NR-SOB in oil field waters P and C (method A) gave much lower counts when applied to produced water from oil field N. Either the NR-SOB in the latter field appeared to be much slower growing or sulfide was removed abiotically over a long period of time. This meant that longer incubation times or the application of method B was required, resulting in much higher counts than were obtained by method A after 30 d of incubation. In addition, this investigation demonstrated that sulfide removal was much faster in the produced waters from oil fields P and C compared to the produced water from oil field N. In the former two waters, the HNRB were stimulated by nitrate amendment, whereas in the latter water, the HNRB were not stimulated by nitrate amendment. These results suggest that, in order to hasten sulfide removal, an active HNRB population is required to either out-compete heterotrophic SRB for carbon source and, or produce end products, from nitrate reduction, that raise the redox potential and inhibit SRB. Both of these processes, performed by HNRB, would disrupt anaerobic sulfur cycling. Further studies are required to specifically prove this hypothesis.

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5. Evaluating a most probable number method for enumerating planktonic dissimilatory ammonium-producing nitrate-reducing bacteria in oil field waters.

5.1 Introduction

Numerous laboratory investigations (Reinsel et al. 1996; Jenneman et al. 1996; Davidova et al. 2001; Chapter 3 and Eckford and Fedorak 2002b; Greene et al. 2003), some field studies (Jenneman et al. 1999; Larsen 2002) and full-scale field operations (Thorstenson et al. 2002) have demonstrated that the microbial formation of H_2S in oil field waters can be controlled by nitrate amendment to these waters. Nitrate stimulates a diverse group of bacteria, known as nitrate-reducing bacteria (NRB), that controls the production of sulfide by sulfate-reducing bacteria (SRB). The NRB use NO_3^- as an electron acceptor for dissimilatory nitrate reduction that is carried out by denitrifying bacteria (DNB), including chemolithotrophic and heterotrophic bacteria that perform respiratory denitrification, and by dissimilatory ammonium-producing-NRB (DAP-NRB). The abbreviation DNRA (for dissimilatory nitrate reduction to ammonia) has been widely used by others (Tiedje 1988; Bonin 1996; Kelso et al. 1997) to describe the activities of the latter group of bacteria.

The DAP-NRB generally have a fermentative metabolism and produce NH_4^+ from NO_3^- in excess of the reduced nitrogen needed for growth (Tiedje 1988). Simon (2002) refers to two ammonium-producing processes as respiratory nitrite ammonification with electron transport and fermentative nitrite ammonification with substrate level phosphorylation. Examples of DAP-NRB include *Clostridia*, rumen bacteria, *Veillonella*, *Desulfovibrio*, *Enterobacteriaceae*, *Photobacterium fischeri*, *Bacillus* (several strains) (Tiedje 1988), *Sulfurospirillum deleyianum* (Eisenmann et al. 1995), *Staphylococcus carnosus* (Neubauer and Gotz 1996), *Vibrio* spp. (Bonin 1996) and *Denitrovibrio acetiphilus* (Myhr and Torsvik 2000). During dissimilatory nitrate reduction, the first reaction is respiratory nitrate reduction where NO_3^- is reduced to NO_2^-. The next step can be divided into two possible routes. One route, performed by DNB, proceeds as follows:

$NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$. The other route, performed by DAP-NRB, proceeds as follows: $NO_2^- \rightarrow NH_4^+$ and possibly N_2O (Zumft 1997).

The presence of chemolithotrophic nitrate-reducing, sulfide-oxidizing bacteria (NR-SOB) (Telang et al. 1997; Gevertz et al. 2000; Chapter 3 and Eckford and Fedorak 2002b) and heterotrophic NRB (HNRB) (Chapter 3 and Eckford and Fedorak 2002b) have been reported in oil field waters and these groups of NRB are stimulated by nitrate amendment (Telang et al. 1997; Chapter 4 and Eckford and Fedorak 2002c). However, there are no reports of the presence or activities of DAP-NRB in oil field waters. The major objectives of this study were to use a most probable number (MPN) method to enumerate DAP-NRB in oil field waters and to determine their numerical importance after NO_3^- supplementation in laboratory microcosms that contained oil field waters. DAP medium (formulation I, described later) was used at the same time as three other media for enumerating NRB (Chapters 3, 4 and Eckford and Fedorak 2002b, 2002c). However, the results from the DAP medium were not reported previously because it was important to evaluate its utility prior to reporting these results.

Generally, tryptic soy broth has been the recommended substrate for DAP-NRB because it is NO_3^- limited (Tiedje 1988). However, the use of this growth medium does not permit distinction between DNB and DAP-NRB (Fazzolari et al. 1989) and the NH_4^+ formed in this medium could be a result of deamination, not dissimilatory nitrate reduction to NH_4^+. Thus, a defined medium was prepared to enumerate DAP-NRB by scoring MPN cultures for ammonium production.

In previous studies, MPN methods were used to enumerate different types of NRB from various oil field waters in Alberta and Saskatchewan, Canada (Chapter 3 and Eckford and Fedorak 2002b) and determine which bacterial populations were affected by the addition of NO_3^- to oil field waters (Chapter 4 and Eckford and Fedorak 2002c). This study reports the evaluation of the DAP medium, the abundance of DAP-NRB in oil field waters and their response to nitrate amendment.

5.2 Materials and methods

5.2.1 Reference bacteria, incubation conditions and analytical methods used for the enumeration of DAP-NRB

The reference bacteria used in this study were a denitrifying bacterium, *Pseudomonas stutzeri*, and two DAP-NRB, *Escherichia coli* and *Citrobacter freundii*. Growth of DAP-NRB and ammonium production were evaluated using a 3-tube MPN procedure. The methods for anaerobic medium preparation and medium inoculation have been described previously (Chapter 2 and Eckford and Fedorak 2002a). Briefly, water samples or suspensions of reference bacteria were diluted to 10^{-11} using dilution blanks prepared with sterile, anaerobic phosphate buffer (10 mM, pH 7.2). These were inoculated by syringe into sealed 16 x 125 mm Hungate type anaerobic culture tubes (Bellco Glass Inc., Vineland, NJ) containing anaerobic medium. The phosphate buffer and media were prepared with Cl^- concentrations to match the corresponding Cl^- concentrations in the oil field water samples given in Chapter 3 by Eckford and Fedorak (2002b). The temperatures of the oil field waters used in this study ranged from 20 to 30°C (Chapter 3 and Eckford and Fedorak 2002b), and all of the cultures were incubated at room temperature (21°C), in the dark. After 28 d of incubation, the culture tubes were observed for growth as seen by turbidity. The MPN index based on turbidity was used as a guide to determine which culture tubes were to be tested for NH_4^+ concentration using the indophenol blue spot test (Feigl and Anger 1972).

Many oil field waters contain NH_4^+ (Collins 1975). Thus, although the media formulations were ammonium-free, inoculation with ammonium-containing oil field water might give false positives based on results from the spot test. Ammonium standards, made from NH_4Cl with concentrations ranging from 0.06 to 2.8 mM, were used with the spot test to semi-quantitatively estimate the NH_4^+ concentrations in the oil field water samples and in the culture tubes after the incubation was complete. To be considered positive for DAP-NRB growth, the medium in a MPN tube had to contain >0.06 mM NH_4^+ and have more NH_4^+ than would be expected from the 10-fold serial dilutions of the ammonium-containing oil field water. Statistical comparisons of the MPN results were done using the method of Cochran (1950).

5.2.2 Evaluation of various carbon substrates and media formulations for enumerating DAP-NRB

Four different media formulations were tested for enumerating DAP-NRB. These were inoculated with dilutions of a wastewater sample collected from the Gold Bar Wastewater Treatment Plant in Edmonton. It was presumed that the sample taken from the biological nutrient removal process, in which nitrification and denitrification occurs, would provide a substantial number of DAP-NRB. Table 5.1 shows the four formulations of media that were tested with different carbon sources and NO_3^- concentrations. The mineral medium base for the four formulations contained per liter: 1.2 g Na_2HPO_4, 1.8 g KH_2PO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.03 g $CaCl_2$, 0.02 g $FeCl_3$, 0.02 $MnSO_4$, 0.5 g $NaHCO_3$ and 10 mL of trace metal solution. The trace metal solution was made in 10.5 mM nitrilotriacetic acid (adjusted to pH 6.0 with KOH) and contained per liter: 1.0 g $MnSO_4 \cdot 2H_2O$, 0.2 g $CoCl_2 \cdot 6H_2O$, 0.5 g $Fe(SO_4) \cdot 7H_2O$, 0.2 g $ZnSO_4 \cdot 7H_2O$, 0.02 g $CuCl_2 \cdot 2H_2O$, 0.02 g $NiCl_2 \cdot 6H_2O$, 0.02 g $Na_2MoO_4 \cdot 2H_2O$, 0.02 g Na_2SeO_4, 0.02 g Na_2WO_4. The pH of the medium was from 6.8 to 7.0. Formulations II and IV also contained 10 mL of anaerobically prepared filter-sterilized vitamin solution per liter of medium. The vitamin solution contained per liter of distilled, deionized water: 2.0 mg biotin, 2.0 mg folic acid, 10 mg pyridoxine HCl, 5.0 mg riboflavin, 5.0 mg thiamine, 5.0 mg nicotinic acid, 5.0 mg calcium pantothenate, 5.0 mg cyanocobalamine, 5.0 mg *p*-aminobenzoic acid, and 5.0 mg thioctic acid.

In addition to the four formulations, each of the seven carbon sources was added individually to the mineral base and tested along with formulations I to IV, using the wastewater sample. The concentration of each carbon source for the individual tests was the same as for formulation III and 10.5 mM NO_3^- was added. Formulation I (designated DAP medium) was superior to the other three formulations for enumerating DAP-NRB, and this formulation was evaluated further.

Table 5.1 Compositions of four media tested for enumerating DAP-NRB in a wastewater sample and the MPN results based on turbidity and ammonium production

Formulation	Carbon sources[a] and concentration (mM)	KNO_3 (mM)	Molar substrate C to NO_3^- ratio	MPN mL^{-1} (95% confidence interval)	
				Based on turbidity	Based on NH_4^+ production
I	Glucose (7.6), Pyruvate (7.3), Succinate (7.4), Acetate (7.3), Glycerol (7.6)	8.4	16	$9.3x10^4$ ($1.5x10^4$ to $38x10^4$)	$4.3x10^4$ ($0.7x10^4$ to $21x10^4$)
II	Glucose (7.6), Pyruvate (15), Succinate (11), Acetate (23), Glycerol (15), Lactate (15), Malate (11), Vitamins	3.4	94	$2.3x10^4$ ($0.4x10^4$ to $12x10^4$)	$2.9 x10^3$ ($1.0x10^3$ to $15x10^3$)
III	Glucose (7.6), Pyruvate (7.3), Succinate (7.4), Acetate (7.3), Glycerol (7.6) Lactate (7.6), Malate (7.6)	12	16	$2.1x10^4$ ($0.35x10^4$ to $47x10^4$)	$1.5x10^3$ ($0.3x10^3$ to $4.4x10^3$)
IV	Glucose (7.6), Pyruvate (15), Succinate (11), Acetate (23), Glycerol (15), Lactate (15), Malate (11), Vitamins	73	4.4	$9.3x10^4$ ($1.5x10^4$ to $38x10^4$)	No MPN index[b]

[a] Acids added as sodium salts.

[b] Ammonium was detected in the MPN tubes but no usable MPN index could be obtained

5.2.3 Comparison of DAP medium and lactose broth for the enumeration of C. freundii *and* E. coli.

The newly developed DAP medium was compared with a standard medium used for the enumeration of coliforms. Lactose broth is used for the detection of lactose-fermenting bacteria, Gram-negative coliforms (Atlas 1995). Difco™ lactose broth (Becton Dickinson, Sparks, MD) was prepared anaerobically by sparging with O_2-free N_2 while dispensing into Hungate tubes.

C. freundii and *E. coli* were grown overnight on Plate Count Agar (Becton Dickinson), then a few colonies were picked and suspended into 10 mL of anaerobic phosphate buffer (pH 7.2) to give an OD_{600} of 0.3. To minimize possible nutrient carry-over from the Plate Count Agar, the cell suspensions were diluted 10-fold in buffer. Then, ten-fold serial dilutions of these cell suspensions were each inoculated to DAP medium and lactose broth. The 3-tube MPN cultures in DAP medium or lactose broth were examined weekly for turbidity. On day 28, ammonium production was determined in DAP medium using the indophenol blue spot test.

5.2.4 Evaluation of NO_3^- conversion to NH_4^+ in DAP medium using ^{15}N analysis

Some of the oil field water samples contained NH_4^+. This NH_4^+ could be detected in the lowest dilution MPN tubes by the indophenol blue spot test after the tubes had been incubated. To confirm that the NH_4^+ in the lowest dilution MPN tubes was actually from DAP-NRB, reducing NO_3^- to NH_4^+, the gas chromatography-mass spectrometry (GC-MS) method developed by Köster and Jüttner (1999) was used. Briefly, for each sample, two sets of DAP medium were prepared and inoculated. One set contained only $K^{14}NO_3$ and the other contained $K^{14}NO_3$ and $K^{15}NO_3$ (MSD Isotopes, Merck Frosst Canada Inc. 99% in excess) in a 3:1 ratio. After 1 month of incubation, selected MPN tubes that had been inoculated with 1 mL of water sample in 10 mL of medium and had NH_4^+ that was detected by the indophenol blue spot test, were analyzed for the presence of $^{15}NH_3$.

Samples from these tubes were derivatized (Köster and Jüttner 1999) and analyzed by GC-MS using a Hewlett Packard 5890 series II GC with a 5970 series mass selective detector and a 30-m DB-5 capillary column (J&W Scientific, Folsom CA). The oven temperature program was: 1 min isothermal at 180°C, 10°C min^{-1} up to 250°C which was held for 3 min. The most abundant fragment ion, *m/z* 284, was compared to the fragment ion *m/z* 285, in order to calculate the at% excess ^{15}N as outlined by Köster and Jüttner (1999). Comparisons were made between cultures with and without $^{15}NO_3^-$ enrichment to determine the at% excess ^{15}N. If the culture contained more ^{15}N than would be expected from naturally occurring isotopic abundance, it was evident that $^{15}NO_3^-$ was reduced to $^{15}NH_3$ by DAP-NRB.

5.2.5 Enumeration of planktonic DAP-NRB in oil field water samples using DAP medium

Eighteen water samples from five oil fields (designated A, B, C, N and P) in Alberta and Saskatchewan, Canada were collected (Table 5.2). Oil field P was sampled twice and these samples were designated Pa and Pb. The various oil field locations and sampling procedures have been described previously (Chapter 3 and Eckford and Fedorak 2002b). The samples were transported to the laboratory on ice, stored at 4°C and inoculated to DAP medium within 24 h.

5.2.6 Enumeration of DAP-NRB in oil field waters after nitrate amendment

Laboratory-scale nitrate-amendment tests were done on one produced water sample from each of three oil fields C, Pa and N (Chapter 4 and Eckford and Fedorak 2002c). The experiments used serum-bottle microcosms containing oil field water supplemented with 10 mM NO_3^-. One purpose of the nitrate-amendment test was to enumerate and monitor changes in various types of NRB, including DAP-NRB, in the microcosms. During the NO_3^- addition study, samples were removed from the microcosms and were tested for chemical and bacterial changes over a 38-d incubation period. Details of the nitrate-amendment study have been previously described (Chapter 4 and Eckford and Fedorak 2002c).

Table 5.2 Oil field water samples used in this study and MPN results for planktonic DAP-NRB and HNRB

Oil field designation and location of sampling	Sample Code	NH_4^+ concentration (mM) in sample[a]	DAP-NRB (MPN mL^{-1}) based on turbidity	DAP-NRB (MPN mL^{-1}) based on NH_4^+ production[a]	HNRB (MPN mL^{-1})[b]
A, Storage tanks	A3	>2.8	<0.3	<0.3	4.3
B, Wellhead PW[c]	B1	<0.06	4.3	<0.3	2.3
B, Treater	B2	<0.06	93	15	4300
B, Storage tanks	B3	<0.06	23	2.3	930
B, Source water	B4	<0.06	43	<0.3	43
C, FWKO[d]	C2	0.3	930	23	430
N, Wellhead PW	N1	0.1	<0.3	<0.3	<0.3
N, FWKO	N2	0.3	23	9.3	93
N, Storage tanks	N3	0.1	230	43	23000
N, Source water	N4	<0.06	210	<0.3	2300
Pa, Satellite[e] PW	Pa1	0.6	<0.3	<0.3	<0.3
Pa, FWKO	Pa2	1	2.3	2.3	1.5
Pa, Storage tanks	Pa3	1	93	23	7.5
Pa, Source water[f]	Pa4	0.6	9300	9300	1500
Pb, Satellite PW	Pb1	0.3	7.5	7.5	2.3
Pb, FWKO	Pb2	0.6	9.3	9.3	43
Pb, Storage tanks	Pb3	0.3	1500	1500	2300
Pb, Source water[f]	Pb4	0.06	230	230	1.5

[a] Ammonium detected with the indophenol blue spot test. Range for test is 0.06 to 2.8 mM NH_4^+

[b] MPN tube scored positive if the NO_3^- concentration decreased in growth medium. Data from Chapter 3 and Eckford and Fedorak (2002b).

[c] PW, produced water

[d] FWKO, free water knock out

[e] A satellite is a collecting point for oil and water directly from the wellheads of several producing wells.

[f] These source waters were actually produced waters from another oil field that were being injected into oil field P.

5.3 Results

5.3.1 Evaluation of various carbon sources and media formulations for enumerating DAP-NRB

Because of its proximity and ease of sampling, wastewater from the biological nutrient removal bioreactor at the local wastewater treatment plant was used to evaluate the four different media formulations for enumerating DAP-NRB. Table 5.1 shows the MPN results based on turbidity and ammonium production. Formulations I and III were made so that essentially the same number of moles of each carbon source was added. Formulations II and IV were made so that the same number of moles of carbon in each carbon source was added. Based on turbidity, the four formulations gave counts ranging from 2.1×10^4 to 9.3×10^4 mL^{-1} (Table 5.1), and there was no statistical differences among these MPN values ($P<0.05$). However, the MPN values based on ammonium production were significantly different.

With formulation I, the MPN results based on turbidity and ammonium production were the same ($P<0.05$) at 9.3×10^4 mL^{-1} and 4.3×10^4 mL^{-1}, respectively (Table 5.1). The amount of NH_4^+ produced in the MPN tubes gave concentrations that were easily determined by the indophenol blue spot test. The reference bacteria grew well in this medium. As expected, *P. stutzeri* did not produce NH_4^+, whereas *E. coli* and *C. freundii* produced NH_4^+.

Using formulation II, the MPN result based on turbidity (2.3×10^4 mL^{-1}) was statistically the same ($P<0.05$) as the result based on ammonium production (2.9×10^3 mL^{-1}, Table 5.1). Based on ammonium production, the MPN result obtained with formulation I was 15-times higher and not statistically the same as that obtained with formulation II ($P<0.05$). The amounts of NH_4^+ produced in formulation II were low, so there were some difficulties detecting NH_4^+ with the spot test. The reference bacteria grew well in the medium. As expected, *P. stutzeri* did not produce NH_4^+ and *E. coli* and *C. freundii* produced NH_4^+.

With formulation III, the MPN result (Table 5.1) based on turbidity (2.1×10^4 mL^{-1}) was 14-times higher and not statistically the same ($P<0.05$) as the result based on ammonium production (1.5×10^3 mL^{-1}). The NH_4^+ produced in the tubes gave concentrations that were easily detected by the spot test. Compared to the results from formulation I, formulation III gave approximately a 29-fold lower MPN value based on ammonium production and these MPN values were not statistically the same ($P<0.05$). The reference bacteria grew poorly in formulation III. *P. stutzeri* and *C. freundii* did not produce NH_4^+ and *E. coli* did.

With formulation IV, the MPN result was 9.3×10^4 mL^{-1} based on turbidity (Table 5.1). However, an MPN index based on ammonium production could not be determined because the NH_4^+ results were scattered throughout the dilution series. The reference bacteria grew poorly in the medium. *P. stutzeri* and *C. freundii* did not produce NH_4^+ and *E. coli* did.

There is no obvious explanation as to why the reference bacteria grew poorly in formulations III and IV. The control bacteria grew well in formulations I and II with NO_3^- concentrations of 8.4 mM and 3.4 mM, respectively. Formulations III and IV had higher NO_3^- concentrations of 12 mM and 73 mM, respectively (Table 5.1).

Seven different media were prepared using the mineral medium plus each of the seven individual carbon sources. These were inoculated with serial dilutions of the same domestic wastewater sample as used to evaluate formulations I to IV. Glucose, succinate and glycerol produced MPN results where turbidity and ammonium production were equal ($P<0.05$). Glucose, pyruvate and succinate had MPN numbers for DAP-NRB that were statistically the same ($P<0.05$). The MPN result based on ammonium production for glycerol was 10-fold higher and not statistically the same ($P<0.05$) as was obtained for the other carbon sources. Glycerol produced growth in higher dilutions than any of the other substrates, and glycerol was a component in all four formulations (Table 5.1).

Of course, there is an infinite number of combinations of carbon sources and carbon to nitrogen ratios that could be tested. However, the results showed that formula I (designated DAP medium) gave the highest counts of DAP-NRB in the wastewater sample, and this formulation was considered satisfactory for enumerating DAP-NRB.

5.3.2 Comparison of DAP medium and lactose broth for the growth of C. freundii *and* E. coli

Liquid medium containing lactose is used routinely for enumerating coliforms (such as *C. freundii* and *E. coli*) to determine the quality of potable water (Eaton et al. 1995). Thus, lactose broth and the DAP medium were compared to see if they would give the same MPN values for suspensions of these two reference bacteria. Both media were prepared anaerobically and inoculated using the same dilution blanks containing the DAP-NRB, lactose-fermenting *C. freundii* or *E. coli*. Growth and ammonium production were observed in MPN tubes of DAP medium inoculated with cell suspensions that were over a million-fold dilutions of the cells picked from cultures grown on Plate Count Agar. Thus, it is highly unlikely that nutrients or NH_4^+ could have been carried over from the Plate Count Agar.

For *E. coli,* the MPN value (1.5×10^8 mL^{-1}) based on turbidity in lactose broth remained the same after 7 d of incubation. In contrast, the MPN value based on turbidity in the DAP medium increased from 4.3×10^6 mL^{-1} on day 7 to 2.3×10^8 mL^{-1} on day14, with no increase thereafter. Comparison of the results from the lactose broth and the DAP medium indicated that, from days 14 to 28, the MPN results based on turbidity were the same ($P<0.05$). Ammonium production was measured only once and that was after the 28 d incubation. From these results, the MPN value based on ammonium production in DAP medium was the same for *E. coli* as the MPN value based on turbidity in lactose broth ($P<0.05$).

In both the DAP medium and lactose broth, the MPN values based on turbidity for the *C. freundii* suspension reached 9.3×10^8 mL^{-1} after 7 d of incubation. No increase in MPN was observed when the MPN tubes were incubated for 28 d. After 28 d, the culture tubes were tested for ammonium production in DAP medium. The MPN value based on ammonium production was the same ($P<0.05$) as was obtained for turbidity with *C. freundii* in lactose broth. These comparisons with the two reference bacteria showed that enumeration with lactose broth and the newly developed DAP medium gave the same results.

5.3.3 Evaluation of NO_3^- conversion to NH_4^+ in DAP medium using ^{15}N analysis

If a water sample contains a high concentration of NH_4^+, and if NH_4^+ is detected only in the lowest dilutions of a MPN series, scoring the MPN tubes is confounded. The NH_4^+ detected in the low dilution MPN tubes may simply be from the water sample and not from the growth of DAP-NRB producing NH_4^+ in the tubes. If NH_4^+ is detected in the MPN tube, its concentration must be estimated so that the MPN tube can be scored positive or negative. Thus, the initial concentration of NH_4^+ in the water sample must also be estimated using the spot test. For example, oil field water Pa2 contained 1 mM NH_4^+ (Table 5.2). Inoculation of 1 mL of this water into 10 mL of ammonium-free medium gave an NH_4^+ concentration of about 0.09 mM. After incubation of the MPN tubes, the concentration of NH_4^+ was >2.8 mM in each of the three MPN tubes that contained 1 mL of this water. These tubes were clearly scored positive for DAP-NRB. Similarly, sample Pb2 contained 0.6 mM NH_4^+ (Table 5.2), and 1 mL of this water into 10 mL of ammonium-free medium gave an NH_4^+ concentration of about 0.05 mM. Again, after incubation of the MPN tubes, the three MPN tubes with 1 mL of oil field water contained 0.6, 0.3 and 0.3 mM NH_4^+. These tubes were also scored positive for growth of DAP-NRB.

The conversion of $^{15}NO_3^-$ to $^{15}NH_4^+$ was used to confirm the presence of DAP-NRB in the lowest dilutions of DAP medium inoculated with three samples collected for this work. One sample was from the wastewater treatment plant and the others were from oil fields N and P. In the undiluted samples, the NH_4^+ concentrations were below detection (<0.06 mM), 2.8 and 2.8 mM, respectively. These samples were serially diluted and inoculated into two sets of DAP medium; one with 25% $K^{15}NO_3$ plus 75% $K^{14}NO_3$ and one with only $K^{14}NO_3$.

Based on ammonium production, the numbers of DAP-NRB in the wastewater sample were not statistically different and these were 2.3×10^6 mL^{-1} in the non-enriched DAP medium and 7.5×10^5 mL^{-1} in the $^{15}NO_3^-$-enriched DAP medium. The MPN tubes with the lowest dilution (1 mL of sample in 10 mL of medium) were chosen for GC-MS analyses. The tubes contained 0.28 mM NH_4^+ for both non-enriched and $^{15}NO_3^-$-enriched wastewater samples. The GC-MS results showed that the NH_4^+ in the $^{15}NO_3^-$-enriched medium had an at% excess ^{15}N equal to 23%, clearly demonstrating that $^{15}NH_4^+$ production from $^{15}NO_3^-$ occurred.

The FWKO water from oil field N had 39 DAP-NRB mL^{-1} in the non-enriched DAP medium and 75 DAP-NRB mL^{-1} in the $^{15}NO_3^-$-enriched DAP medium. These MPN values were not significantly different (*$P<0.05$*). The FWKO water from oil field P had 64 DAP-NRB mL^{-1} in the non-enriched DAP medium and 93 DAP-NRB mL^{-1} in the $^{15}NO_3^-$-enriched DAP medium, and these MPN values were not different from each other (*$P<0.05$*). The MPN tubes with 1 mL of undiluted sample in 10 mL of medium were chosen for GC-MS. For the sample from oil field N, the tubes contained 0.4 and 2.8 mM NH_4^+ for the non-enriched and $^{15}NO_3^-$-enriched media, respectively. For the sample from oil field P, the tubes contained 0.6 and 0.8 mM NH_4^+ for the non-enriched and $^{15}NO_3^-$-enriched media, respectively. GC-MS analyses showed that the NH_4^+ in the $^{15}NO_3^-$-enriched medium inoculated with sample N or P had an at% excess ^{15}N equal to 23%. Estimating the NH_4^+ concentrations with the spot test suggested that there was more NH_4^+ present in these MPN tubes than the concentration that originated from dilution of the oil field water samples. Dilution of the water samples with no activity of DAP-NRB would give a NH_4^+ concentration of 0.3 mM. The detection of NH_4^+ enriched in ^{15}N unequivocally showed that DAP-NRB were active in these MPN tubes.

5.3.4 Survey of numbers of DAP-NRB in oil field water samples

Eighteen oil field samples were collected and used to enumerate planktonic DAP-NRB with the DAP medium. Table 5.2 summarizes the MPN values based on turbidity and ammonium production. Table 5.2 also contains the MPN results for HNRB in these same samples. The HNRB results were reported previously (Table 3.2 and Eckford and Fedorak 2002b). Only three (samples A3, N1 and Pa1) of the 18 samples showed no turbidity in the DAP medium, and these MPN values were reported as <0.3 mL^{-1}. Similarly, these three samples showed no ammonium production, and the DAP-NRB MPN result for each was given as <0.3 mL^{-1}. Three additional samples (B1, B4 and N4) gave MPN values of <0.3 mL^{-1} based on ammonium production, although turbidity was observed in the tubes of DAP medium. To improve the detection limit of any MPN procedure, larger sample volumes can be inoculated into appropriate volumes of medium with proportionally high concentrations of nutrients. However, this procedure was not used in the current study.

Of the 12 oil field water samples that produced NH_4^+ in DAP medium (Table 5.2), 10 had the same ($P<0.05$) MPN results regardless of whether the tubes were scored on turbidity or on ammonium production. Thus, using the presence of turbidity to choose which tubes to test for NH_4^+ by the spot test was quite reliable. The majority (9 of 12) of the water samples that produced NH_4^+ had DAP-NRB numbers that were less than 50 mL^{-1}. The highest counts were found in samples from oil field P from the storage tanks (sample Pb3) and the "source water" (samples Pa4 and Pb4). However, this "source water" was actually sour, produced water from another nearby oil field that was delivered to oil field P for disposal by injection into the formation.

A dilute nutrient broth-nitrate medium (Chapter 3 and Eckford and Fedorak 2002b) was used to enumerate the HNRB. To block denitrification at N_2O (Tiedje 1982), acetylene (25% v v^{-1}) was added to the headspace of each culture tube. After incubation, the MPN tubes were scored for NO_3^- consumption and N_2O production. *P. stutzeri*, *E. coli* and *C. freundii* grew in this medium, consuming NO_3^- and producing N_2O (Chapter 3 and Eckford and Fedorak 2002b).

Planktonic HNRB were detected in 16 of 18 oil field samples based on NO_3^- loss in dilute nutrient broth-nitrate medium (Table 5.2). The two samples with no detectable HNRB in dilute nutrient broth-nitrate medium (samples N1 and Pa1) also had no detectable DAP-NRB. Only one sample (Pb4) contained more DAP-NRB than HNRB ($P<0.05$), and the numbers of DAP-NRB and HNRB were the same in six samples (all from oil field P; Pa2, Pa3, Pa4, Pb1, Pb2, Pb3). In five samples (B2, B3, C2, N2, and N3), the numbers of HNRB were greater than the numbers of DAP-NRB. In four samples (A3, B1, B4 and N4), there were HNRB and no DAP-NRB (Table 5.2).

5.3.5 The responses of various types of NRB after nitrate amendment to oil field water

In laboratory studies, oil field waters Pa3, N3 and C2 were amended with 10 mM NO_3^-. DAP-NRB were enumerated using DAP medium at the same time that samples were taken for the enumeration of HNRB and NR-SOB in nitrate-amended and unamended microcosms (Table 4.3 and Eckford and Fedorak 2002c). The results for sample Pa3 are summarized in Figure 5.1. Counts were done on days 0 and 38.

In the nitrate-amended microcosm, the DAP-NRB MPN increased 15-fold, from 23 mL^{-1} on day 0 to 350 mL^{-1} on day 38. The MPN values on day 0 and on day 38 based on turbidity and ammonium production in DAP medium were statistically the same (*P<0.05*) for the nitrate-amended microcosm (Figure 5.1). In the unamended microcosm, the number of DAP-NRB was 43 mL^{-1} on day 38, which was not significantly different from the day 0 count (*P<0.05*).

In contrast, the number of HNRB increased 57,000-fold to 4.3x10^5 mL^{-1} after 38 d in the nitrate-amended microcosm (Table 4.3 and Figure 5.1). Similarly, NO_3^- supplementation stimulated the NR-SOB, and their numbers increased 2900-fold, from

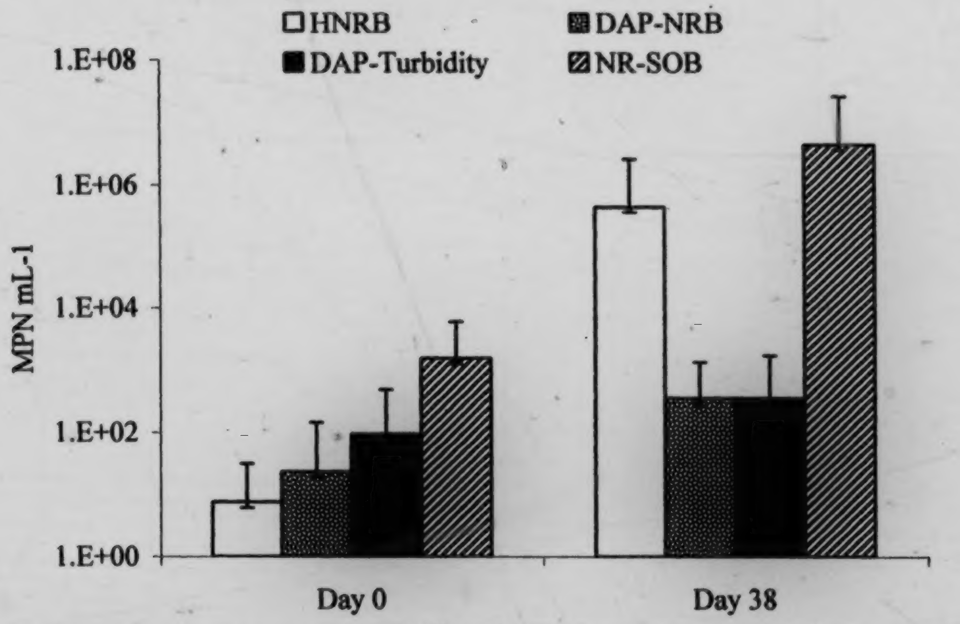

Figure 5.1 Numbers of HNRB, DAP-NRB (based on ammonium production), growth in DAP medium (based on turbidity) and NR-SOB in microcosms that contained oil field water Pa3 amended with 10 mM NO_3^-. The error bars indicate the 95% confidence interval of the MPN values. Data for the HNRB and NR-SOB are from Table 4.3 and Eckford and Fedorak (2002c).

1.5×10^3 to 4.3×10^6 after 38 d incubation (Table 4.3 and Figure 5.1). In the unamended microcosm, the MPN value for the HNRB increased 12-fold by day 38, while there was no significant increase in the numbers of NR-SOB (Table 4.3). Nitrate was depleted from this microcosm by day 14 (Figure 4.1 and Eckford and Fedorak 2002c).

To better understand the dynamics of the changes in numbers of NRB, the microcosms that contained oil field water samples N3 or C2 were analyzed and enumerated at 7 to 10 d intervals, with a total incubation time of 38 d. The results for samples N3 and C2 are summarized in Figures 5.2 and 5.3, respectively. In 38 d the initial 10 mM NO_3^- was decreased to about 3 mM for oil field water N3 (Figure 4.4) and 5 mM for oil field water C2 (Figure 4.2).

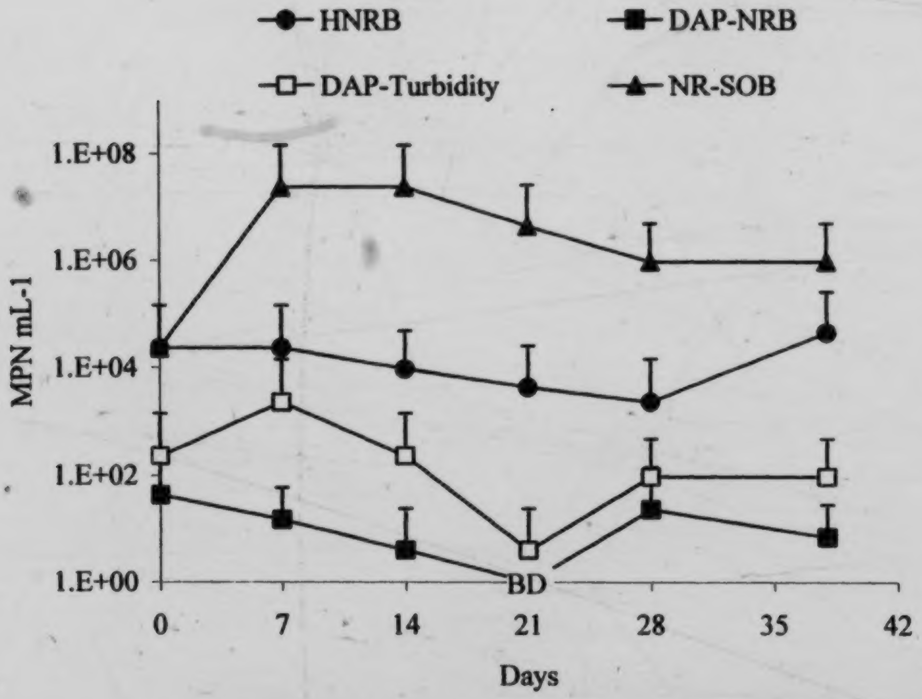

Figure 5.2 Numbers of HNRB, DAP-NRB (based on ammonium production), growth in DAP medium (based on turbidity), and NR-SOB in microcosms that contained oil field water N3 amended with 10 mM NO_3^-. For DAP-NRB on day 21, the MPN result was below detection (BD) (<0.3 MPN mL^{-1}). Data for the HNRB and NR-SOB are from Chapter 4 and Eckford and Fedorak (2002c). The error bars indicate the 95% confidence interval of the MPN values.

Initially, the numbers of HNRB and NR-SOB were 530-fold higher than the number of DAP-NRB in oil field water N3 (Figure 5.2). After nitrate amendment there was no statistically significant increase for the HNRB and the DAP-NRB over the testing period. The NR-SOB increased 1000 times to 2.3×10^{7} mL^{-1} by day 7 (Table 4.2) There was little change in the numbers of NRB in the microcosm that was not supplemented with NO_3^- (Figure 4.5).

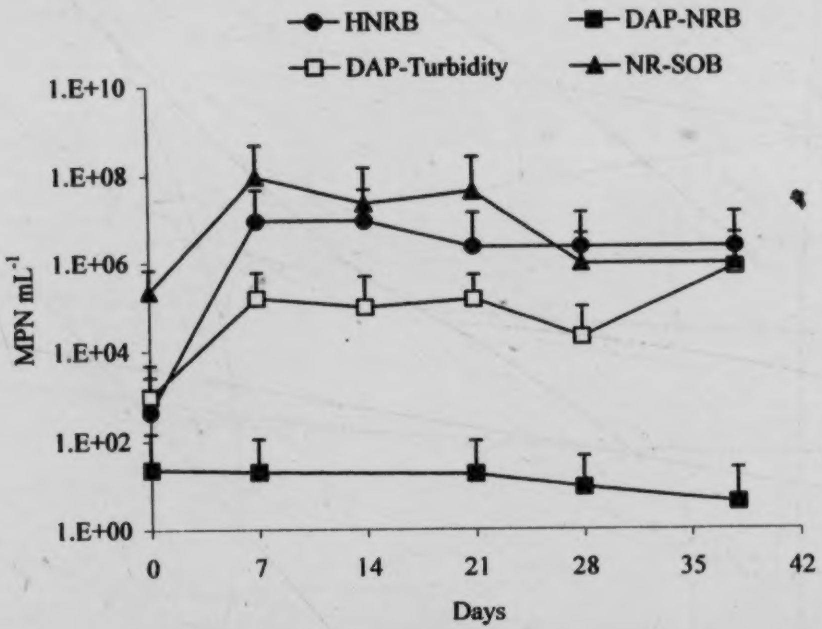

Figure 5.3 Numbers of HNRB, DAP-NRB (based on ammonium production), growth in DAP medium (based on turbidity), and NR-SOB in microcosms that contained oil field water C2 amended with 10 mM NO_3^-. The error bars indicate the 95% confidence interval of the MPN values. Data for the HNRB and NR-SOB are from Chapter 4 and Eckford and Fedorak (2002c).

Throughout the 38-d incubation, the numbers of DAP-NRB were much lower than the numbers of the other two types of NRB in the nitrate-amended oil field water C2 (Figure 5.3). At day zero, the numbers of HNRB and NR-SOB were about 19-fold and 9000-fold, respectively, higher than the number of DAP-NRB. In the presence of NO_3^-, the numbers of HNRB and NR-SOB increased 22,000-fold and 440-fold respectively, during the first 7 d of incubation (Table 4.2). However, there was no increase in the number of DAP-NRB during the 38-d incubation (Figure 5.3). Again, there was little change in the numbers of NRB in the microcosms that were not amended with NO_3^- (Figure 4.3).

Also given in Figures 5.2 and 5.3 are the MPN results based on the turbidity that appeared in the DAP-medium (shown as open squares). In many cases, turbidity was observed in tubes that were inoculated with high dilutions of the sample, but no NH_4^+ was detected in these tubes. This gave MPN values based on turbidity that were greater ($P<0.05$) than the MPN values based on ammonium production. This is discussed in more detail later.

5.4 Discussion

The activities of NRB in anaerobic environments can aid in the control of undesirable substances. For example, in domestic wastewater treatment facilities, NRB play a key role in the nutrient removal process by reducing NO_3^- to N_2 before the effluent is released (Robertson and Kuenen 1992). Also, NO_3^- addition at oil field production facilities stimulates NRB, and this method has been shown to control the production of H_2S (Jenneman et al. 1999; Thorstenson et al. 2002; Larsen 2002). The numbers of HNRB and NR-SOB have been shown to increase after NO_3^- addition to oil field waters (Chapter 4 and Eckford and Fedorak 2002c), but no studies have focused on the activities of DAP-NRB. The main aims of this study were to enumerate heterotrophic DAP-NRB in oil field waters and determine their relative importance in nitrate-amended waters. To do this, a MPN method was developed for enumerating DAP-NRB.

More than one carbon source was included so that diverse populations of DAP-NRB could be cultivated from environmental samples. The carbon sources tested included: glucose and glycerol for fermentation (Akunna et al. 1993), pyruvate for sulfate-reducing bacteria with ammonium-producing capabilities (Widdel and Pfennig 1984), malate for *Veillonella* sp. that produce NH_4^+ (Yordy and Delwiche 1979), acetate for oil field isolates like *Denitrovibrio acetiphilus* (Myhr and Torsvik 2000), succinate for *Wolinella succinogenes* (Bokranz et al. 1983) and lactate as used by Bonin (1996) for cultivating dissimilatory nitrate reducers. Of course, during fermentation, end products that can be formed include fatty acids, alcohols and H_2 which could be used as electron donors by bacteria in the sample (Gottschalk 1988).

One of the challenges in developing a medium to enumerate DAP-NRB was to ensure that there was enough NO_3^- in the medium so that if dissimilatory ammonium production occurred, the resulting concentration of NH_4^+ would be detected by the spot test used to score the MPN tubes. The DAP medium contained 8.4 mM NO_3^-. Various NO_3^- concentrations in media used for the cultivation of DAP-NRB have been reported. Examples of NO_3^- concentrations are: Samuelson (1985) used 3 mM; Bonin (1996), Fazzolari et al. (1989), and Yin et al. (2002) used 10 mM and Smith (1982) used 1, 5 and 10 mM. Indeed, the NO_3^- concentration in the DAP medium falls in the range reported by others.

Dissimilatory nitrate reduction to NH_4^+ is favored when NO_3^- is limiting (Tiedje et al. 1982; Kelso et al. 1997). In natural environments under low oxygen conditions, nitrate reduction to N_2 or NH_4^+ can occur. Which process is favored may depend on habitat as well as the carbon to nitrogen ratio. Tiedje et al. (1982) mention that it could be energetics and kinetics which explain the nitrate reduction process that occurs in a particular environment. Energy yield per electron donor (H_2) provides DNB with more potential energy, and energy available per NO_3^- consumed would favor DAP-NRB, because eight electrons can be accommodated when ammonium production occurs and five electrons when denitrification occurs. It appears as though many DAP-NRB posses fermentative capabilities, so that anaerobic environments, which select for fermenters and obligate anaerobes, would favor DAP-NRB. Likewise, aerobic environments would select for DNB because they compete efficiently for carbon under respiratory conditions.

Tiedje et al. (1988) postulated the "the ratio of available carbon-to-electron-acceptor controls whether NO_3^- partitions to DAP-NRB or to denitrification". In other words, during metabolism, the greatest need when the environment has a high carbon to electron acceptor ratio is maximum electron acceptor capacity, and the greatest advantage when the environment has low carbon to electron acceptor ratio is for DNB that gain the most energy per NO_3^-. This carbon to electron acceptor ratio hypothesis is supported by studies from trout farming (Christensen et al. 2000) and shellfish farming (Gilbert et al. 1997) as well as from anaerobic sediments (Kelso et al. 1997).

A variety of elevated carbon to nitrogen ratios have been used in previous studies to stimulate DAP-NRB. For example, Smith (1982) used molar ratios of glucose-C to NO_3^- from 24 to 240 in batch cultures with defined medium to grow a soil *Citrobacter* sp. Samuelsson (1985) used a molar glucose-C to NO_3^- ratio of 14 when testing the marine bacterium *Pseudomonas putrefaciens* for the production of NH_4^+. Kelso et al. (1997) showed that a molar glucose-C to NO_3^- ratio of 37 produced more NH_4^+ than a ratio of 4, and Bonin (1996) showed that more NH_4^+ was produced in cultures with molar glucose-C to NO_3^- ratios of 24 and 120 compared to 2.4 and 12. In this study, three of the four media formulations that were tested had carbon to nitrogen molar ratios of 16 or greater (Table 5.1).

Formulation I was superior to the other formulations when evaluated with a domestic wastewater sample that contained DNB (data not shown) and DAP-NRB. With formulation I (DAP medium), the MPN results based on turbidity or ammonium production were the same ($P<0.05$), and the MPN value for ammonium production was higher than for any other formulation. In addition, the reference DAP-NRB produced NH_4^+ concentrations that were easily detected by the indophenol blue spot test. The fact that formulations I to IV yielded the same amount of growth, based on turbidity ($P<0.05$), indicated that anaerobic heterotrophs grew equally well in all formulations.

For the enumerations of two DAP-NRB, *E. coli* and *C. freundii*, the DAP medium gave the same MPN values as the widely used lactose broth. These two media are based on different growth characteristics of these two reference bacteria. The ability to ferment lactose is the basis for enumeration in lactose broth, whereas the ability to carry out dissimilatory nitrate reduction to NH_4^+ is the basis for enumeration in the DAP medium. Based on turbidity, *E. coli* grew more slowly in the DAP medium than in the lactose broth. However, the MPN values were the same after 14 d of incubation. The intent was to incubate DAP medium containing oil field waters for 28 d (as was done with the other media to enumerate various NRB) so the differences in growth rate of *E. coli* would not be noticed during routine work.

Ammonium is commonly found in oil field waters. Collins (1975) tabulated data from nearly 100 different formation waters, and NH_4^+ concentrations ranging from 0 to 2660 mg L^{-1} (148 mM) were found in the waters. Most waters contained a few hundred milligrams of NH_4^+ per liter (10 to 20 mM). All but one of the oil field samples contained $\leq$1 mM NH_4^+ (Table 5.2).

The ability of the MPN method to detect low numbers of DAP-NRB in waters that contain high concentrations of NH_4^+ has not been tested. However, the presence of high concentrations of NH_4^+ from a diluted oil field water would make it difficult to distinguish NH_4^+ produced by DAP-NRB from NH_4^+ in the water sample. Thus, it would be difficult to correctly score a MPN growth tube positive or negative. It was clearly demonstrated that measuring the reduction of $^{15}NO_3^-$ to $^{15}NH_4^+$ can be used to detect the activities of DAP-NRB in the DAP medium, but this isotope method would be far too costly and labor-intensive for routine use to determine MPN results. Because of the low NH_4^+ concentrations in the oil field water samples, false positives were not encountered in this study.

Although dissimilatory nitrate reduction to NH_4^+ has been widely studied, there appears to be only one study in which defined medium, devoid of organic nitrogen, was used to enumerate DAP-NRB. Bonin (1996) used a MPN method with artificial sea water, without NH_4Cl, and supplemented with 5 g glucose L^{-1} and 1 g KNO_3 L^{-1} to enumerate DAP-NRB in coastal marine sediments. Media that were free of organic nitrogen have been used by others (Fazzolari et al. 1989, 1990; Myhr and Torsvik 2000) to cultivate, but not enumerate, DAP-NRB.

The method for enumerating DAP-NRB relies on detecting ammonium production in the medium. Thus, the DAP-medium was formulated without NH_4^+. A consequence of this choice is that the DAP-NRB may have difficulty initiating growth and producing NH_4^+. During studies in which DAP-NRB were isolated from coastal marine sediments, Bonin (1996) observed that cells grew very slowly in ammonium-free medium, and during the first 3 d of incubation little or no NH_4^+ was released. This led Bonin (1996) to state that the rate of NH_4^+ assimilation seemed to be higher than the rate of nitrate reduction to NH_4^+. However, some of her isolates produced NH_4^+ in as little as 12 h (depending on the composition of the defined growth medium). Fazzolari et al. (1990) studied the anaerobic growth of *Enterobacter amnigenus*, a DAP nitrate-reducing bacterium, in defined medium that was devoid of NH_4^+. They observed that ammonium production and accumulation began during the late log phase. The results from the *E. coli* enumeration, comparing lactose broth and DAP medium, showed that the 14-d incubation in DAP medium was needed before the MPN result, based on turbidity in DAP medium, reached the MPN result after a 7 d incubation in lactose broth. The longer incubation time required for the DAP medium was likely due, in part, to the ammonium-free formulation of the medium. Nonetheless, the 28-d incubation time should minimize any biases caused by the slow onset of growth of DAP-NRB in ammonium-free medium.

Denitrification by DNB and ammonium production by DAP-NRB may occur simultaneously in an environment. Whether denitrification or ammonium production is the dominant nitrate-reducing activity depends on the conditions in the environment. Gilbert et al. (1997) studied a water-sediment interface in an area used for shellfish farming. When comparing the shellfish farming site to an area outside the site, they noted that the organic content and reduction state of the sediments determined the fate of nitrogen. The farming site generally had a low redox potential so reduction processes like denitrification and ammonium production occurred. The area outside the farming site had positive redox potential values so oxidation processes like nitrification occurred. Seasonal increases of the C/N ratio in the sediment due to sedimentation of summer phytoplanktonic production, favored ammonium production for both areas. Denitrification was favored corresponding to seasonal inorganic nitrogen inputs from the land. In the shellfish farming site, ammonium production accounted for 98% of the NO_3^- reduced.

Bonin et al. (1998) found that both nitrate reduction processes affected coastal sediments and that variability for each process had more to do with available carbon than with NO_3^-, oxygen and pH. Stevens et al. (1998) studied the effects of soil pH on nitrate reduction to nitrogen gases. They found that NO_2^- accumulation and DAP-NRB are favored at a pH of 8.0. Brundel and Garcia-Gil (1996) investigated nitrate reduction in anaerobic freshwater sediments and found that the inhibition of NO- and N_2O-reductase by 1 mM H_2S was thought to channel electrons to produce NH_4^+. Sulfide toxicity to denitrification in a shallow estuary was also reported (An and Gardner 2002).

It is not known whether DNB or DAP-NRB are active in oil field waters. Some of the environmental conditions in the oil fields that were sampled would be conducive to DNB or DAP-NRB activities. For example, the pH values of waters from oil fields C, N and P ranged from 7.5 to 9.0 (measured at atmospheric pressure) and the water temperatures were between 20 and 30°C (Table 3.2 and Eckford and Fedorak 2002b). In addition, organic acids and hydrocarbons dissolved in the formation waters would provide an ample supply of carbon (Head et al. 2003). However, these oil fields are souring because of microbial sulfide production, and the sulfide concentrations range from 0.2 to 5 mM (Table 3.2 and Eckford and Fedorak 2002b). Although these concentrations may inhibit heterotrophic denitrification, Eisenmann (1995) reported that the facultative *S. deleyianum* could grow with "sulfide (up to 5 mM) as electron donor, nitrate as electron acceptor, and acetate as carbon source". Most importantly, the lack of NO_3^- would preclude any nitrate-reducing activity. Collins (1975) reported that "it is unlikely that petroleum-associated waters contain appreciable amounts of NO_3^-".

Nonetheless, several different types of NRB have been detected in many oil field water samples (Adkins et al. 1992; Gevertz et al. 2000; Davidova et al. 2001; Table 3.2 and Eckford and Fedorak 2002b), and nitrate-reducing activities can be stimulated by NO_3^- supplementation (Telang et al. 1997; Davidova et al. 2001; Chapter 4 and Eckford and Fedorak 2002c; Thorstenson et al. 2002). Typically, the numbers of NRB and other bacteria are low in wellhead samples (Adkins et al. 1992; Table 3.2 and Eckford and Fedorak 2002b). This is also true of the numbers of DAP-NRB (Table 5.2). In three of the samples (designated wellhead or satellite) no DAP-NRB were detected (samples B1, N1, and Pa1). The term satellite is used to describe a collection point where oil from several wellheads are gathered and pumped through a single pipeline to a treater or FWKO. Higher numbers of DAP-NRB were observed in samples from aboveground facilities, such as the treater, FWKO and storage tanks (Table 5.2). These same samples often contained high numbers of aerobic bacteria (Table 3.2 and Eckford and Fedorak 2002b). These environments may have given respiring HNRB an advantage over the fermentative DAP-NRB. The MPN values in eight samples from aboveground facilities (A3, B2, B3, B4, C2, N2, N3 and N4) showed that the numbers of HNRB were greater than the numbers of DAP-NRB (Table 5.2).

Attached microbial growth and biofilms are common in oil field habitats and these biofilms harbor huge numbers of microorganisms. Typically, the numbers of planktonic microorganisms are small compared to those in biofilm (McInerney and

Sublette 2002). However, there was no attempt to collect biofilm samples; instead, water samples, that were easier to collect, were studied. Thus, the data in Table 5.2 summarizes only the numbers of planktonic DAP-NRB and HNRB from the various oil field sites. It is very likely that NRB are more abundant on the solid surfaces in the oil field environment.

Microcosms containing oil field waters were monitored to determine which types of NRB were stimulated by nitrate amendment (Figures 5.1 to 5.3). Chapters 3 and 4 (Eckford and Fedorak 2002b, 2002c) describe different MPN procedures that were used to enumerate DAP-NRB and HNRB. The MPN results for HNRB, determined on the basis of NO_3^- loss and N_2O production, were the same ($P<0.05$) for all the water samples that grew in the nutrient broth-nitrate medium. Nitrate-reducing bacteria that produce N_2O include DNB and DAP-NRB (Tiedje 1988), and *E. coli* and *C. freundii* grow and produce N_2O in the nutrient broth-nitrate medium. The medium that was used to enumerate HNRB was not prepared anaerobically so that the facultative nitrate-respiring NRB would have an advantage. In contrast, the DAP medium was anoxic, which would favor anaerobes.

The data in Figures 5.1 to 5.3 suggest that the DAP-NRB were not stimulated in the microcosms, but these results are confounded by the appearance of turbid cultures beyond the dilutions in which NH_4^+ was detected. This phenomenon was observed with the microcosms inoculated with oil field waters N3 (Figure 5.2) and C2 (Figure 5.3) but it was not observed with the microcosms inoculated with oil field water Pa3 (Figure 5.1). The MPN results based on turbidity are shown as open squares in Figures 5.2 and 5.3. There are three situations that could account for growth in these higher dilutions: (1) HNRB grew in the medium without producing NH_4^+ (as did the test organism *P. stutzeri*), (2) heterotrophs unable to reduce NO_3^- consumed all of the NH_4^+ produced by the DAP-NRB or (3) and combination of (1) and (2). Situation (1) would have little or no effect on the DAP-NRB MPN results based on the measurement of ammonium production. However, situations (2) and (3) would result in MPN tubes being scored falsely negative because all of the NH_4^+ that was produced, was consumed. This would yield falsely low MPN values for the DAP-NRB.

Based on dry weight, a typical bacterial cell contains about 50% carbon and 14% nitrogen (Stanier et al. 1970), so the cell has a C/N ratio of 3.6. On a mass basis, the DAP medium has a C/N ratio of about 13. Thus, there is an adequate supply of carbon in the DAP medium to allow complete assimilation of the NH_4^+ produced by the DAP-NRB, as suggested in situation (2).

Forty water samples used in this study were grouped according to their sources (Table 5.3). Growth in the DAP medium, based on turbidity, was observed with all of the samples except for three oil field waters. The last column of Table 5.3 shows the number of samples that produced MPN results, based on turbidity, that were statistically higher than the MPN results, based on NH_4^+ detection. This occurred least frequently (1 of 7 samples) with wastewater and river water samples and most frequently (8 of 11 samples) with waters from the nitrate-amended microcosms.

Table 5.3 Summary of the numbers of samples that may have given falsely low MPN values for DAP-NRB because growth yielded turbidity in the medium at dilutions beyond which NH_4^+ was detected

Sample type	Numbers of samples[a]	Numbers of samples showing turbidity in DAP medium	Numbers of samples for which the MPN result, based on turbidity, was greater than the MPN result, based on detected NH_4^+ ($P<0.05$)
Wastewater and river water	7	7	1
Oil field waters	22	19	6
Waters from nitrate-amended microcosms	11	11	8

[a] These include some samples that were not discussed elsewhere in this paper.

These observations point out a limitation of this MPN method. That is, the MPN value obtained in the DAP medium might be an underestimation of the culturable number of DAP-NRB if all of the produced NH_4^+ was assimilated by other microorganisms in the MPN tubes. Although the assimilation of all the produced NH_4^+ seems unlikely, there is no simple analysis of nitrogen-containing compounds (such as NO_3^-, NO_2^-, N_2O or NH_4^+) in the spent MPN medium that can resolve this issue. Even tracing the fate of ^{15}N from $^{15}NO_3^-$ (which would be far too laborious for a MPN method) would not solve this dilemma because none of the possible fates of the ^{15}N would be unique to the DAP-NRB. Thus, the results from this MPN method must be used with caution when microbial growth produces turbidity in the medium at dilutions beyond which NH_4^+ is detected.

Although no simple chemical analysis can resolve situations in which falsely low MPN values might be obtained because other microorganisms consume all of the NH_4^+ produced by the DAP-NRB in MPN tubes, molecular biology methods are likely applicable. For example, Michotey et al. (2000) evaluated MPN-PCR and competitive PCR methods to enumerate denitrifying bacteria. They successfully used primers that were specific for *nirS* gene encoding for cytochrome *cd*$_1$ found in many denitrifying bacteria. Applying the Cochran statistical test to their data, indicates that the molecular methods were superior to the traditional culturing MPN method for enumerating NRB in a sediment sample, but the molecular methods gave the same results as the traditional MPN method for a water sample ($P<0.05$).

The key enzyme in the reduction of NO_2^- to NH_4^+ is cytochrome *c* nitrite reductase (Simon 2002). Thus, it should be possible to design a primer specific for a gene encoding this reductase and to use this primer with the MPN-PCR or competitive PCR methods to specifically enumerate DAP-NRB. There appears to be no study that has attempted this.

The results obtained from the DAP medium while monitoring the nitrate-amended microcosms (Figures 5.2 and 5.3) gave ambiguous MPN values because turbidity was observed in the medium at dilutions beyond which NH_4^+ was detected. These data can be considered as either of two extreme cases. In the first extreme case, the true MPN values for the DAP-NRB are given by the results that are based on the detection of NH_4^+ in the medium. These results give lower counts, and these MPN values are shown as solid squares in Figures 5.2 and 5.3. In this case, the turbidity observed in the medium at dilutions beyond which NH_4^+ was detected would have likely been caused by HNRB growing in the DAP-medium. In the second extreme case, each tube of medium that showed turbidity would have contained DAP-NRB and other heterotrophs that consumed all of the produced NH_4^+. The results from this unlikely case would give higher counts, and these counts are shown as open squares in Figures 5.2 and 5.3.

Considering the results shown as solid squares in Figures 5.2 and 5.3, leads to the conclusion that the DAP-NRB were not stimulated during nitrate amendment and their numbers remained low throughout the incubation period. Considering the results shown as open squares, could lead to the conclusion that the numbers of DAP-NRB increased in the presence of NO_3^-, but the DAP-NRB numbers were usually much smaller than those of the NR-SOB or the HNRB. For example, the day 7 sample in Figure 5.2 shows the number of NR-SOB (triangle) to be about 10,000-fold higher than the number of DAP-NRB (open square). Similarly, the day 7 sample in Figure 5.3 shows the number of NR-SOB (triangle) to be about 1000-fold higher than the number of DAP-NRB (open square). Overall, either set of results obtained from the DAP medium suggests that the DAP-NRB were far less abundant than other types of NRB in these microcosms.

The work of Telang et al. (1997) is the only study where DAP-NRB in oil field waters were considered. Those authors evaluated the effect of nitrate injection on the microbial community in the same oil field location C that was used in the study. Telang et al. (1997) monitored the bacterial changes in the produced water using the technique known as reverse sample genome probing (RSGP). One of the DNA standards on their master filter was from an oil field isolate (designated NH15b) that was tentatively identified as a *Citrobacter* sp. or *Salmonella* sp. These genera reduce NO_3^- to NH_4^+, thus this DNA standard represented some DAP-NRB. After NO_3^- addition to oil field C, Telang et al. (1997) observed that there was no increase in the relative abundance of bacterial DNA that hybridized with the NH15b DNA. The results from the DAP medium (shown as solid squares in Figure 5.3) corroborate the observations of Telang et al. (1997).

Schürmann et al. (2003) used $^{15}NO_3^-$ to study NO_3^- consumption in an anoxic petroleum-contaminated aquifer (containing 2.4 mg total hydrocarbons L^{-1}). Solutions of 0.34 and 0.35 mM $^{15}NO_3^-$ were injected into the contaminated aquifer, and then on 3 consecutive days, water was extracted from the injection well, and analyzed to determine the fates of the ^{15}N. Denitrification accounted for 46 to 49% of the NO_3^- consumption and dissimilatory nitrate reduction to NH_4^+ accounted for about 4% of the NO_3^- consumption.

In this study, the numbers of DAP-NRB in the microcosms (Figures 5.1 to 5.3) were much lower than the other types of NRB, suggesting that DAP-NRB would not play a major role in NO_3^- consumption in anoxic petroleum-affected water supplemented with NO_3^-. This is consistent with the observations of Schürmann et al. (2003).

In conclusion, DAP medium is suitable for enumerating planktonic DAP-NRB in oil field waters with low NH_4^+ concentrations and in wastewaters and river waters (Table 5.3). Its selectivity is based on ammonium production from NO_3^- in a defined medium without organic nitrogen. However, interpretation of the results from the DAP medium is confounded when there are turbid MPN cultures beyond the dilutions in which NH_4^+ is detected. This was especially evident in the samples from the nitrate-amended microcosms. Although accurate MPN counts were difficult to obtain in some cases, work with the DAP medium suggested that planktonic DAP-NRB are not abundant in oil field waters and that they would remain a minor part of the microbial community after nitrate amendment to the oil field waters.

In summary, more work could be done to investigate the MPN results where turbidity is found in MPN cultures beyond the dilutions in which NH_4^+ is detected. The work could include the development of MPN-PCR molecular biology methods. The results from the molecular MPN-PCR and MPN culturing methods should be compared.

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6. Attempts to determine the role of oil field HNRB in controlling sulfide removal in enrichment cultures and defined co-cultures.

6.1 Introduction

Sulfide produced by oil field sulfate-reducing bacteria (SRB) can be controlled by the addition of NO_3^- to oil field waters. Sulfide depletion in oil field waters has been demonstrated in laboratory studies (Davidova et al. 2001; Chapter 4 and Eckford and Fedorak 2002c; Greene et al. 2003), field studies (Jenneman et al. 1999, Larsen, 2002) and full-scale operations (Thorstenson et al. 2002). Resident nitrate-reducing bacteria (NRB) become active when NO_3^- is added to oil field waters and it is the sulfide-oxidizing, nitrate-reducing bacteria (NR-SOB) that remove sulfide as they use it for an energy source (Gevertz et al. 2000).

The types of oil field NRB that become stimulated after NO_3^- addition are diverse. They can include heterotrophic NRB (HNRB) and chemolithotrophic NRB (Chapter 3 and Eckford and Fedorak 2002b). These bacteria can perform respiratory denitrification which proceeds as follows: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$ (Tiedje 1988).

Many studies have looked at the mechanisms by which sulfide concentrations are controlled in the presence of NO_3^- and $SO_4^=$. Five proposed mechanisms are discussed in section 1.6.1. Some of these mechanisms include competition between HNRB and heterotrophic SRB for a common electron donor, production of N_2O to raise the redox potential and inhibit growth of SRB, removal of sulfide by NR-SOB and production of NO_2^- to inhibit SRB. The studies investigating sulfide depletion in oil field waters have mainly focussed on chemolithotrophic NR-SOB and in particular, *Thiomicrospira* sp. strain CVO from the Coleville, Saskatchewan, Canada oil field. This NR-SOB quickly removes sulfide and becomes the dominant NRB after NO_3^- addition.

HNRB from oil fields have been shown to produce NO_2^- and N_2O in laboratory microcosm studies (Chapter 4 and Eckford and Fedorak 2002c). Wright et al. (1997) studied the effect of adding NO_3^-, water-soluble organics, trace metals and phosphate on the stimulation of oil field bacteria for the oxidation of sulfides in "soured" reservoir brines from oil reservoirs with temperatures ranging from 40 to 60°C. The researchers noted the need for the addition of organic substrates, NO_3^- and yeast extract, in some cases, as well as trace metals and vitamins. They concluded that in west Texas brines, sulfide bioscavenging by indigenous bacteria was mediated by heterotrophic bacteria.

HNRB that produce N_2O have been enumerated from oil field waters (Table 3.2 and Eckford and Fedorak 2002b). Telang et al. (1997) included three heterotrophic isolates on a master filter for reverse sample genome probing (RSGP) analysis. The isolates were able to produce NO_2^- from NO_3^- but were not tested for sulfide oxidation. They were isolated from the Coleville, Saskatchewan oil field which has a moderate temperature and salinity, and oil is produced by water injection. Recently, from the same oil field, two lactate-utilizing NRB strains, NO3A and NO2B, were isolated after produced water was used to inoculate continuous up-flow packed-bed bioreactors. Both strains oxidize lactate with NO_3^-, and strain NO2B can oxidize sulfide when incubated in medium with sulfide, NO_3^- and no lactate (Hubert et al. 2003).

Generally, HNRB that oxidize sulfide are facultative microorganisms (Friedrich 1998). A well characterized example of a HNRB that can oxidize sulfide is *Paracoccus pantotrophus*. This bacterium is classified as a facultative aerobe and facultative chemolithotroph and is capable of mixotrophic and heterotrophic growth on a wide range of substrates, including acetate. It was found in a desulfurizing, denitrifying effluent-treatment system in Delft, Netherlands (Robertson and Kuenen 1983, Rainey et al. 1999). In most cases it is thought that heterotrophic sulfur bacteria do not gain energy from inorganic sulfur compounds. The sulfur transformations are incidental to the major metabolic pathways (Paul and Clark 1996).

In Chapter 4 (Eckford and Fedorak 2002c) it was shown that NO_3^- addition to oil field waters resulted in the removal of sulfide in laboratory microcosms. Produced oil field waters were tested from three locations in Alberta and Saskatchewan, Canada. The results were not the same for the three locations. After 10 mM NO_3^- was added to the oil field waters, the sulfide was removed quickly in two oil field waters and slowly in the third, and the removal of sulfide in the oil field waters was not related to the initial sulfide concentration. The results also showed that two oil field waters had quick sulfide removal with dramatic increases in HNRB numbers and the other oil field water had slow sulfide removal and no change in HNRB number. From this information, a hypothesis was made that HNRB may play a role in the quick removal of sulfide after NO_3^- addition to oil field waters. There appears to have been no study that determined a role for HNRB in sulfide removal from oil field waters after NO_3^- addition. Therefore, this study was done to investigate HNRB in oil field waters from the three oil fields that were previously considered during nitrate amendment. The study was designed to include HNRB and NR-SOB from oil field waters so that the roles for both NRB could be determined during sulfide removal. The SRB, *Desulfococcus multivorans*, was also included. The *D. multivorans* would reduce $SO_4^{=}$ and produce sulfide. When *D. multivorans* was combined with the NR-SOB and HNRB, it was hypothesized that sulfide would be oxidized and NO_3^- would be reduced to NO_2^- and, or N_2O. The NO_2^- and, or N_2O would raise the redox potential of the microcosm and inhibit *D. multivorans*.

6.2 Materials and methods

6.2.1 Strategy used for microcosm studies

The study was designed to use chemolithotrophic NR-SOB, HNRB and heterotrophic SRB in laboratory microcosm studies to determine what roles, other than competition for electron donor, HNRB may have in controlling sulfide production. Three enrichment cultures of chemolithotrophic NR-SOB and three pure cultures of HNRB were obtained from enrichments using three oil field waters (oil fields C, P and N). The chemolithotrophic NR-SOB enrichment cultures were not examined for purity. The SRB, *D. multivorans,* was from a culture collection agency. Acetate and benzoate were chosen as carbon sources for this study because they have been shown to be present in petroleum reservoirs (Magot et al. 2000). The two carbon sources were added in order to prevent competition for a carbon source between the two heterotrophic bacteria. The HNRB were chosen to use acetate as a carbon source, tolerate low concentrations of sulfide (0.8 mM) and produce N_2O from the reduction of NO_3^-. The heterotrophic SRB was chosen to use benzoate as a carbon source, and produce sulfide from the reduction of $SO_4^{=}$.

Microcosm cultures were prepared using a medium which could simultaneously grow HNRB, chemolithotrophic NR-SOB and heterotrophic SRB. The mineral medium, called CSB(2) medium, contained components from two formulations used in other studies. The two formulations were modified CSB medium described in Chapter 3 (Eckford and Fedorak 2002b) and a modified formulation of Widdel Pfennig medium described by Hines et al. (2002). Various concentrations of carbon sources and NO_3^- were used. A low concentration of benzoate (1 mM) was used in order to prevent toxic inhibition of HNRB. Two concentrations of acetate (15 and 25 mM) were chosen so that acetate would not become limiting in microcosm studies with 3 and 10 mM nitrate, respectively. The two concentrations of NO_3^- were used in order to demonstrate NO_3^- limitation for oil field C (3 mM) and no NO_3^- limitation for oil fields P and N (10 mM).

Microcosm studies containing one, two or three types of bacteria were done in CSB(2) medium for each set of enriched oil field cultures. These microcosms were monitored for chemical and bacterial changes. The microcosm studies with one type of bacterium (called single culture microcosms) contained an oil field pure culture of HNRB or an oil field enrichment culture of chemolithotrophic NR-SOB or *D. multivorans*. The microcosm studies with two types of bacteria (called double culture microcosms) contained HNRB plus NR-SOB or HNRB plus *D. multivorans* or NR-SOB plus *D. multivorans*. The microcosm studies with three types of bacteria (called triple culture microcosms) contained an oil field pure culture of HNRB plus an oil field enrichment culture of NR-SOB plus *D. multivorans*. The single culture microcosms allowed for each bacterium or enrichment to be characterized. Each bacterium or enrichment was then monitored when combined with one or two other bacteria in the double and triple microcosms.

6.2.2 Oil field waters for enrichment of HNRB and NR-SOB

The oil field waters used in this study are from locations C, P and N and have been described previously as well as the method of collection for oil field waters from locations N and P (Chapters 3, 4 and Eckford and Fedorak 2002b, 2002c). The isolation of HNRB from oil field waters were done using freshly collected preinjection oil field waters from oil fields C, P and N; scrapings from oil field filters (10 µm and 100 µm) from a preinjection location at oil field N and oil field waters from oil fields C, P and N that had been stored at 4°C under anaerobic conditions for up to 1.5 years. The NR-SOB enrichments were done using oil field waters from locations N and P that had been stored at 4°C under anaerobic conditions for 6 months and a newly collected water sample from oil field C transported via courier to the U of A.

6.2.3 Media for enrichment of oil field HNRB and NR-SOB

For all media described in this study, NaCl, KNO_3, Na_2SO_4, $Na_2S{\cdot}9H_2O$, sodium acetate and sodium benzoate were used. For enrichment of HNRB and NR-SOB, the Cl^- concentration of the oil field water was initially added to the medium used for the enrichment. As culture transfers continued, the Cl^- concentrations ranged from 0.12 M to 0.17 M. Media, except nutrient broth-nitrate liquid medium, were made anaerobically with O_2-free 10% CO_2, balance N_2.

Enrichments for NR-SOB were done in modified CSB medium containing 2.2 mM sulfide, as described in Chapter 3 and Eckford and Fedorak (2002b), and no acetylene was added to block N_2O reduction. The modified CSB medium contained no carbon source and will be referred to as CSB medium hereafter.

Media used for enrichments of HNRB were nutrient broth-nitrate liquid medium, previously described (Chapter 2 and Eckford and Fedorak 2002a) with no acetylene added, and the nutrient broth-nitrate formulation with 15 g agar L^{-1} added for plates. Isolated colonies from plates were inoculated to modified Widdel Pfennig medium. Widdel Pfennig medium, described by Hines et al. (2002), was modified and contained per liter: 7g NaCl, 1.2 g $MgCl_2{\cdot}6H_2O$, 0.3 g NH_4Cl, 4 g Na_2SO_4, 0.2 g KH_2PO_4, 0.3 g KCl, 0.15 g $CaCl_2{\cdot}2H_2O$, 10 mL of 0.1 g L^{-1} resazurin, 960 mL distilled, deionized H_2O (pH 7.0 to 7.5). This solution was autoclaved 20 min at 121°C and maintained sterile while cooled under O_2-free 10% CO_2, balance N_2. Sterile trace metal, selenite and vitamin solutions, described below, were added along with 1 mL of sterile 0.5 M $Na_2S{\cdot}9H_2O$. The medium was dispensed into sterile 16 x 125-mm Hungate-type anaerobic culture tubes (Bellco Glass, Vineland, NJ) flushed with sterile O_2-free 10% CO_2, balance N_2. Where indicated, sterile, anaerobic NO_3^-, sulfide, acetate and benzoate were added later to the medium.

Where indicated, the trace metal and selenite solutions added to media were: 1 mL per liter of a trace metal solution containing per liter 4.5 mL HCl, 1.5 g $FeCl_2 \cdot 4H_2O$, 60 mg H_3BO_3, 100 mg $MnCl_2 \cdot 4H_2O$, 120 mg $CoCl_2 \cdot 6H_2O$, 70 mg $ZnCl_2$, 25 mg $NiCl_2 \cdot 6H_2O$, 15 mg $CuCl_2 \cdot 2H_2O$, 25 mg $Na_2MoO_4 \cdot 2H_2O$ and 1 mL per liter of a selenite solution containing per liter: 3 mg $Na_2SeO_3 \cdot 5H_2O$ and 0.5 g NaOH. Also, where indicated, 10 mL per liter of a vitamin solution, described in section 5.2.2, was added.

6.2.4 Methods for enrichment of oil field HNRB and NR-SOB

Anaerobic technique was used throughout the enrichment process. Transfers of culture and medium were made using syringes flushed with O_2-free N_2. All Hungate-type anaerobic culture tubes used for NR-SOB enrichment were incubated in a Coy anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) as described previously (Chapter 3 and Eckford and Fedorak 2002b).

Enrichment for HNRB from oil field waters included inoculation of oil field water to nutrient broth-nitrate liquid medium and then transfer to fresh medium after the inoculated medium became turbid. In the experimental design, the oil field HNRB isolates were required to use acetate and not benzoate as a carbon source and the cultures from oil field N required the addition of trace metal solutions. The subsequent two transfers for all oil field HNRB enrichment cultures were made to nutrient broth-nitrate liquid medium with 15 mM acetate plus trace metals and vitamins. The enrichments were spread on nutrient broth-nitrate agar plates with acetate, trace metals and vitamins. Isolated colonies were streaked onto fresh nutrient broth-nitrate agar plates. The pure isolates from the agar plates were washed and diluted in phosphate buffer (10 mM, pH 7.2) before inoculation to liquid modified Widdel Pfennig medium containing 15 mM acetate or 3.5 mM benzoate. The tubes were observed for growth in acetate or benzoate as seen by turbidity.

After a 14 d incubation, liquid transfers were made to fresh modified Widdel Pfennig medium containing 15 mM acetate or 3.5 mM benzoate. This was done three times. At the end of the third transfer, the culture tubes that appeared turbid in modified Widdel Pfennig medium with acetate were analyzed for N_2O production (Chapter 2 and

Eckford and Fedorak 2002a). Dinitrogen formation was not blocked by the addition of acetylene. The same isolate grown in modified Widdel Pfennig medium with benzoate was also analyzed for N_2O production.

The HNRB cultures were monitored for sulfide tolerance in modified Widdel Pfennig medium with 15 mM acetate plus 0.8 mM sulfide. After it was shown that the cultures could grow in the presence of 0.8 mM sulfide and acetate, the cultures were transferred from the modified Widdel Pfennig medium to CSB medium containing no carbon source and 0.8 mM sulfide. The reason for determining whether the HNRB would grow in CSB medium with sulfide and no carbon source had to do with the most probable number (MPN) procedures. Briefly, in the double and triple enrichment culture microcosm studies, the HNRB were combined with chemolithotrophic NR-SOB. In order to monitor changes for each bacterium in the microcosms, MPN procedures were used for each bacterial type. It was important to show that the HNRB would not grow in CSB medium that was used for enumerating chemolithotrophic NR-SOB (described in section 6.2.5) and that the MPN procedures for each bacterium could be used with confidence.

For the initial NR-SOB enrichments, 1 mL of oil field water was added to 9 mL of CSB medium and then 10-fold serial dilutions were done from 10^{-1} to 10^{-3} in CSB medium containing 2.2 mM sulfide. Cultures were considered positive, based on color change in the CSB medium, after incubation in an anaerobic chamber. Cultures that turned pink in CSB medium were considered positive for growth with depletion of sulfide. Cultures that turned yellow in CSB medium were considered positive for growth with decrease of sulfide. These color changes are described in section 4.3.4. Further transfers were made to CSB medium containing different sulfide concentrations with and without vitamin and acetate additions. It appeared that vitamin addition did not make any difference to the NR-SOB enrichment cultures. However, vitamins were added to the medium because the *D. multivorans*, used in the study, required vitamins for growth. Cultures were tested for NO_2^- and N_2O production (no acetylene was added for N_2O detection).

6.2.5 Bacteria, media and methods for laboratory microcosm studies

Bacteria, other than the NR-SOB and HNRB oil field enrichments, that were used for laboratory microcosm studies included: *D. multivorans* DSMZ 2059, a SRB that can use benzoate as a carbon source; *Paracoccus pantotrophus* ATCC 35512, a facultative chemolithotroph that can use sulfide as an energy source and acetate as a carbon source; and NR-SOB, *Thiomicrospira* strain CVO (obtained from Dr. Voordouw's laboratory at the University of Calgary), a chemolithotrophic NR-SOB that can oxidize sulfide.

A medium was used that could grow HNRB, NR-SOB and SRB simultaneously. The enrichment studies (sections 6.3.1 and 6.3.2) showed that the CSB mineral base formulation was suitable for the NR-SOB enrichments from the oil field waters and could support growth of the oil field HNRB when a carbon source was added. SRB have been shown to grow in variations of the Widdel and Pfennig formulation (Hines et al. 2002) and the modified Widdel and Pfennig medium used in our laboratory. The formulation, CSB(2) was developed and included ingredients from the CSB and modified Widdel and Pfennig media. The CSB(2) medium contained the same ingredients per liter as CSB medium (Chapter 3 and Eckford and Fedorak 2002b) with these changes: 10 g NaCl, 2 g $MgSO_2{\cdot}7H_2O$, 0.35 g $(NH_4)_2SO_4$, 1 mL selenite solution, 0.1 mL $NiCl_2{\cdot}6H_2O$ (from a 25 mg per 100 mL stock solution). Various concentrations of NO_3^-, $SO_4^=$ and sulfide were added, depending on the study. The selenite solution was described in section 6.2.3. The medium was prepared anaerobically and autoclaved at 121°C for 20 min. When cooled, sterile, anaerobic solutions of sulfide, vitamins (10 mL L^{-1}, described in section 5.2.2.), acetate and benzoate were added from sterile, anaerobic stock solutions. Just before inoculation, media for SRB was reduced with anaerobic $Na_2S_2O_4$ to give a concentration of 10-30 mg L^{-1}.

Bacteria used for the studies were grown in the above medium with different carbon sources, the HNRB with acetate, the SRB with benzoate and the NR-SOB with CO_2. The oil field enrichments did not tolerate the process of centrifugation and subsequent suspension into anaerobic phosphate buffer for the purpose of washing the cells to remove components from the culture medium, so the cultures were maintained in liquid medium. Liquid aliquots were taken from cultures and inoculated to fresh medium.

After the microcosms were inoculated with bacteria, 1.5 mL of sample was removed and filtered through a 0.2 µm pore size Millex-GS Millipore filter (Bedford, MA). The filtered samples were frozen at -20°C and analyzed for NO_3^-, NO_2^-, $SO_4^{=}$, acetate and benzoate. Care was taken to ensure that the sample containers did not overflow and that components were not lost due to precipitation during freezing. On each sampling day, 0.1 mL of sample was removed and analyzed for sulfide using the methylene blue method (CHEMetrics, Calverton, VA). Where indicated, 1 mL of sample was removed on day 0 and then days 7, 14 and sometimes 21. These were diluted in dilution blanks of sterile, anaerobic phosphate buffer (10 mM, pH 7.2, sparged with O_2-free N_2) for MPN enumerations.

MPN procedures (3-tube) were used to monitor bacterial changes as described previously (Chapter 3 and Eckford and Fedorak 2002b). The medium used for HNRB was nutrient broth-nitrate liquid medium with added trace metal and selenite solutions, described above. For NR-SOB, CSB medium was used which had various concentrations of sulfide: 2.2 mM sulfide for NR-SOB from oil field C, 1 mM for NR-SOB from oil field P and 0.8 mM for NR-SOB from oil field N. The medium for SRB was either modified Widdel and Pfennig medium with 3.5 mM benzoate or CSB(2) medium with 3.5 mM benzoate. The SRB medium was reduced with 0.2 mM sulfide and 10-30 mg L^{-1} anaerobic $Na_2S_2O_4$, before inoculation. All MPN tubes were incubated for 30 d at 30°C. The nutrient broth-nitrate liquid medium was incubated in the laboratory ambient air and the CSB and SRB media were incubated in the anaerobic chamber. Growth was determined by turbidity for the HNRB and color change for the NR-SOB as described before. For the SRB MPN tubes, 1 drop of a 5% anaerobic solution of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ was added and the tubes were considered positive based on turbidity and black coloration from FeS formation.

Preliminary microcosm studies were done using *P. pantotrophus* and *Thiomicrospira* strain CVO. As well, microcosm studies were done using the HNRB and NR-SOB oil field enrichments from oil fields C, P and N (Table 6.1). For all microcosm studies, 120-mL serum bottles were used which contained a final volume of 90 mL CSB(2) medium.

Final microcosm studies were done using *D. multivorans* and oil field enrichments HNRB and NR-SOB using CSB(2) medium. The days for analyses and substrate concentrations are shown for each oil field study in Tables 6.2, 6.3 and Figure 6.1 for oil field C; Tables 6.4, 6.5 and Figure 6.2 for oil field P and Tables 6.6, 6.7 and Figure 6.3 for oil field N. The microcosms were set up in triplicate. A fourth microcosm was included for each set of microcosms and contained 25% v v^{-1} of headspace acetylene gas to block nitrate reduction at N_2O. For each oil field, the microcosms were three sets, each inoculated with one type of bacterium (single enrichment cultures), HNRB, NR-SOB or SRB; three sets, each inoculated with two types of bacteria (double enrichment cultures), HNRB plus NR-SOB, HNRB plus SRB or NR-SOB plus SRB; one set inoculated with three types of bacteria (triple enrichment cultures), HNRB, NR-SOB plus SRB. The microcosms were inoculated with 1% v v^{-1} HNRB, 3% v v^{-1} NR-SOB and 5% v v^{-1} SRB. MPN enumerations were done (days 0, 7 and 14) on the microcosm used for chemical analyses. One set of sterile medium control microcosms was set up for each oil field study.

6.2.6 Chemical analyses for laboratory microcosm studies

Due to time restraints, analyses for NO_3^-, NO_2^-, $SO_4^{=}$, acetate and benzoate were done on only one of the triplicate microcosms and N_2O was analyzed on the fourth microcosm containing acetylene. The microcosm observations for color change, turbidity and sulfide analyses were done on all three microcosms. For simplicity, the results in the text, figures and tables are described for one microcosm. The methods for analyses of N_2O by gas chromatography and NO_2^- by colorimetry have been previously described (Chapters 2, 3 and Eckford and Fedorak 2002a, 2002b). Nitrate was measured either by ion chromatography (IC), described previously (Chapter 2 and Eckford and Fedorak 2002a) or by the Cataldo et al. (1975) method as described by Greene (1999). Sulfate was either determined using IC, described previously (Chapter 2 and Eckford and Fedorak 2002a) or by the Kolmert et al. (2000) method.

The high performance liquid chromatography (HPLC) method, used to measure acetate and benzoate, was taken from Miwa (2000) using an Agilent (Wilmington, DE)

1100 series HPLC instrument. HPLC conditions were described by Clemente (2004). The solvent flow rate was 1.2 mL min^{-1} and the mobile phase was 40% HPLC-grade methanol (Fisher Chemicals, Fairlawn NJ) and 60% MilliQ water. Elutions were approximately 1.9 min for acetate and 7 min for benzoate. Forty microliters of derivatized sample was injected onto the column. The samples were derivatized using 50 μL of sample or standard mixed with 100 μL of acidic 2-nitrophenylhydrazine hydrochloride (2-NPH·HCl; TCI America, Portland, OR) and 100 μL of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride (1-EDC·HCl; Sigma Chemicals Co., St. Louis, MO). The acidic 2-NPH·HCl solution was prepared by mixing 75.8 mg 2-NPH:HCl, 7.5 mL 95% ethanol and 2.5 mL 0.2 M HCl. The 1-EDC·HCl solution was prepared by mixing 479.3 mg 1-EDC·HCl, 10 mL 95% ethanol and 10 mL 3% (v v^{-1}) pyridine in ethanol. The reaction mixture was put into sealed 1.5-mL screw-capped vials and incubated in a water bath at 60°C for 20 min. Vials were removed from the water bath and 50 μL of 69 mM KOH (made in 80% v v^{-1} methanol-water) was added. The vials were incubated again in the water bath for 15 min at 60°C and then removed and cooled in an ice bath before HPLC analyses. Standard curves for acetate from 0.1 mM to 20 mM and benzoate from 0.1 mM to 5 mM were used to determine the concentrations of acetate and benzoate in the samples.

6.3 Results

6.3.1 HNRB from oil field water enrichments

Forty-six HNRB isolates were obtained from the nutrient broth-nitrate agar plates. Following the third transfer to liquid modified Widdel and Pfennig medium with acetate as the carbon source, eight cultures became slightly turbid and produced N_2O. There was no obvious turbidity and no N_2O production for the same isolates in modified Widdel and Pfennig medium with 3.5 mM benzoate as the carbon source. After further monitoring for sulfide tolerance, five of the eight cultures were chosen as possible candidates for further studies. When the HNRB isolates were transferred from the modified Widdel and Pfennig medium to CSB medium with no acetate and 0.8 mM sulfide, the CSB medium containing 0.8 mM sulfide remained unchanged for all the oil field HNRB isolates after 30 d.

One HNRB isolate was chosen from each oil field water. The isolates were named HNRB-P, HNRB-N and HNRB-C, with reference to the oil field water where they originated. The HNRB will be given these designations hereafter. The isolates for N and P were enriched from stored oil field waters and the isolate for C was from a freshly collected oil field water. The isolates preferred 30°C incubation temperature and became turbid within one week in liquid medium when maintained in CSB(2) medium with acetate as a carbon source. They grew best in nutrient broth-nitrate liquid medium, were obligate heterotrophs, used acetate as a carbon source, tolerated up to 0.8 mM sulfide and produced N_2O from nitrate reduction. When the isolates were inoculated into modified Widdel and Pfennig medium containing 15 mM acetate plus 3.5 mM benzoate, the cultures did not become turbid. They were then inoculated into modified Widdel and Pfennig medium with 15 mM acetate and 1.8 mM benzoate. The medium became turbid, indicating that the HNRB isolates were able to tolerate this concentration of benzoate.

The HNRB isolates were also screened using 10 mM of carbon sources butyrate, propionate, valerate, malate, succinate and lactate. All three isolates showed slight turbidity using valerate, succinate and lactate as carbon sources after 30 d. Due to time restraints, no further studies were done using these carbon sources.

6.3.2 NR-SOB from oil field water enrichments

Oil field waters from oil fields C, P and N were enriched for chemolithotrophic NR-SOB using CSB medium incubated in an anaerobic chamber. Growth of NR-SOB in the CSB medium was scored as described in section 4.3.4. Briefly, CSB medium contains the redox indicator, resazurin, which is colorless when reduced and pink when oxidized. When sulfide is removed by NR-SOB, the medium turns pink because the redox potential is increased by the presence of the nitrate-reduction products, NO_2^- and, or N_2O. When sulfide is partially removed by NR-SOB, the medium turns yellow. The yellow color may be due to the nitrate-reduction product NO_2^-, as shown in the Appendix. In all cases, cultures that were pink in CSB medium were considered positive for growth with depletion of sulfide and cultures that were yellow in CSB medium were considered positive for growth with decrease of sulfide. The NR-SOB from the oil field enrichment cultures were not checked for purity. One NR-SOB enrichment culture was chosen from each oil field water. The enrichments were named NR-SOB-P, NR-SOB-N and NR-SOB-C, with reference to the oil field water where they originated. The NR-SOB will be given these designations from this point.

6.3.2.1 NR-SOB-C

The initial enrichment for NR-SOB, using oil field water C, became pink in all four CSB dilution tubes within 7 d. The addition of acetate did not appear to make any difference in the cultivation of the enrichments, so acetate was not added to the medium. For all transfers, 2.2 mM sulfide in CSB medium was used and the medium became pink within 7 d. At no time did the CSB medium become turbid. Once the medium in the 10^{-3} dilution tube, from the initial enrichment, turned pink, 10-fold serial dilutions were made to 10^{-9} in CSB medium containing 2.2 mM sulfide. The inoculum for the dilutions came from the 10^{-3} dilution tube. The CSB tubes turned pink in all dilution tubes within 7 d. The culture in the 10^{-9} dilution tube was serially diluted again to 10^{-9} in CSB medium containing 2.2 mM sulfide. Again, all the CSB tubes turned pink within 7 d. The culture in the 10^{-9} CSB tube from the second dilution series was used for further transfers to fresh medium.

Analyses for NO_2^- and N_2O on CSB NR-SOB-C cultures showed that both were formed. Transfers were made to nutrient broth-nitrate plus trace metals liquid medium and agar plates (incubated in the laboratory ambient air) to determine if heterotrophic bacteria were present. Neither turbidity nor colonies were observed in these transfers. The NR-SOB-C was continually transferred to fresh CSB medium containing 2.2 mM sulfide. There was no visible change in the culture after many transfers and the sulfide-containing medium turned pink very rapidly after each transfer.

6.3.2.2 NR-SOB-P

The initial inoculation of oil field water P into CSB medium containing 2.2 mM sulfide resulted in all four CSB dilution tubes forming a yellow color by 14 d. The P water was again inoculated to CSB medium containing 0.5 mM and 1 mM sulfide. In 10 d, the CSB medium containing 0.5 mM sulfide turned pink and the CSB medium containing 1.0 mM sulfide showed no change. The culture from the CSB medium containing 0.5 mM sulfide was transferred to various CSB media containing 2.2 mM sulfide, 1 mM sulfide, acetate and vitamins. The acetate did not make any obvious difference in the cultivation of the NR-SOB and the CSB tubes with both sulfide concentrations became pink by 4 d.

Two sets of 10-fold serial dilutions to 10^{-9} were made in CSB medium containing 2.2 mM sulfide, using the culture in CSB medium containing 1 mM sulfide as the inoculum. In the first dilution series, the CSB tubes became pink to the 10^{-7} dilution tube and yellow in the 10^{-8} and 10^{-9} dilution tubes in 4 d. The 10^{-7} pink CSB culture was used as the inoculum for the second series of dilutions. For the second dilution series, in 5 d the CSB tubes became pink to the 10^{-8} dilution tube. The 10^{-8} CSB culture from the second dilution series was transferred to fresh CSB medium with 2.2 mM or 1 mM sulfide. The CSB medium with 2.2 mM sulfide became yellow in 11 d and the CSB tube with 1 mM sulfide became pink in 3 d. The pink culture in CSB medium with 1 mM sulfide was used for subsequent transfers.

Analyses of NO_2^- and N_2O on CSB NR-SOB-P cultures showed that only NO_2^- was formed. Acetylene had not been added to block N_2O reduction. Transfers were made to nutrient broth-nitrate plus trace metals liquid medium and agar plates (as for NR-SOB-C) to determine if heterotrophic bacteria were present. Neither turbidity nor colonies were observed in these transfers. The NR-SOB-P was continually transferred to fresh CSB medium containing 1 mM sulfide. There was no change in the culture after many transfers and the sulfide-containing medium turned pink very rapidly after each transfer.

6.3.2.3 NR-SOB-N

After 14 d, the initial cultures from the oil field N water into CSB medium with 2.2 mM sulfide resulted in the CSB medium in the first dilution tube turning pink and the CSB medium in the 10^{-1} to 10^{-3} dilution tubes turning yellow. The CSB medium tube that had turned pink was used as the inoculum for 10-fold serial dilutions. The first set of dilutions were made to 10^{-9} in CSB medium containing acetate plus 2.2 mM sulfide and CSB medium containing only 2.2 mM sulfide. In 4 d, the CSB medium containing only sulfide turned pink in all dilution tubes and the CSB medium containing acetate and sulfide were colorless in the 10^{-1} and 10^{-2} dilution tubes and pink in all the other tubes.

A second set of dilutions was made to 10^{-9} in CSB medium containing only 2.2 mM sulfide, using the 10^{-9} culture from the first dilution set with CSB medium plus 2.2 mM sulfide, as the inoculum. All the CSB dilution tubes turned yellow in 5 d. Subsequent transfers of the 10^{-9} CSB culture tube, used as the inoculum for the second set of dilutions, were made to fresh CSB medium with 2.2, 1 and 0.5 mM sulfide. In all cases, the CSB tubes turned yellow or remained unchanged. The next step was to transfer the 10^{-3} and 10^{-6} CSB dilution tubes, from the first set of dilutions with CSB medium containing only 2.2 mM sulfide, to fresh CSB medium containing 0.5 mM or 1 mM sulfide. In 4 d these transfers turned the medium pink. Transfers were continued from these CSB culture tubes to fresh CSB and modified Widdel and Pfennig media with 0.5 mM, 0.8 mM and 1 mM sulfide.

Analyses for NO_2^- and N_2O on CSB NR-SOB-N cultures showed that only NO_2^- was formed in the culture. Acetylene had not been added to block N_2O reduction. Transfers were made to nutrient broth-nitrate plus trace metals liquid medium and agar plates (as for NR-SOB-C). In this case, vitamins and acetate were also added to the medium. These cultures were used to determine if heterotrophic bacteria were present in the enrichment culture. Neither turbidity nor colonies were observed in these transfers.

After the culture had been transferred many times, the types of media and concentrations of sulfide that the enrichment culture could tolerate were determined. The transfer did not show consistent pink color changes in CSB or modified Widdel and Pfennig media. As time progressed, it became evident that the NR-SOB-N could not tolerate sulfide concentrations greater than 0.8 mM and would not turn the medium pink. As a result, 2-month old enrichment cultures in modified Widdel and Pfennig and CSB media were transferred to fresh CSB medium, formulation CSB(2).

The enrichment culture NR-SOB-N was obtained from the transfer. It was from CSB medium with 0.8 mM sulfide transferred to CSB(2) medium with 0.8 mM sulfide. At first, the NR-SOB-N could turn the CSB(2) medium pink within a week. Later, the culture became inconsistent upon transfer to fresh medium and turned the CSB(2) medium pink or yellow from 1 week to 1 month or showed no color change at all. The medium did not become turbid with NR-SOB-N.

6.3.3 Preliminary studies with single enrichment microcosms

Initial microcosm studies were done to screen for the types of NR-SOB and HNRB that could be used for microcosm studies containing more than one type of bacteria. *P. pantotrophus* was chosen because it is a HNRB that anaerobically grows on acetate and not benzoate, uses NO_3^- and oxidizes sulfide (Robertson and Kuenen 1983). *Thiomicrospira* strain CVO was chosen because it is an obligate chemolithotrophic NR-SOB (Gevertz et al. 2000). *P. pantotrophus* and *Thiomicrospira* strain CVO were inoculated to microcosms containing modified Widdel and Pfennig medium and 3 mM benzoate, 10 mM acetate, 20 mM $SO_4^=$, 20 mM NO_3^- and 0.9 mM sulfide. When incubated in the medium, as single culture microcosms, the *P. pantotrophus* and *Thiomicrospira* strain CVO grew well, removed sulfide and used NO_3^-.

Another microcosm study was done in which *P. pantotrophus* and *Thiomicrospira* strain CVO were incubated together. The results from the combined microcosm study showed that it was difficult to determine the role for each bacterium in the microcosm and difficult to enumerate each bacterium using MPN procedures. The *P. pantotrophus* oxidized sulfide and grew in the MPN medium used to enumerate *Thiomicrospira* strain CVO. After reviewing the results from these initial microcosm studies, it was concluded that HNRB and NR-SOB from each oil field, described in sections 6.3.1 and 6.3.2, would be used for further studies.

Single culture microcosm studies using HNRB and NR-SOB from the oil field waters were done in CSB(2) medium. The HNRB isolates and NR-SOB enrichment cultures were incubated alone in the medium. The results are shown in Table 6.1. Sterile medium control microcosms were included. The results for the sterile medium control microcosms showed no change in sulfide, $SO_4^=$, NO_3^-, NO_2^-, acetate and benzoate over the testing period.

For the HNRB and NR-SOB single culture microcosm studies shown in Table 6.1, there was no change in benzoate concentration in any of the microcosms. For each microcosm in Table 6.1, samples were removed for chemical analyses on days 0, 3, 7, 10, 14 and 21. One milliliter of sample was removed for 3-tube MPN procedures on days 0, 7, 14 and 21.

For each HNRB, sulfide was removed, NO_2^- was formed, medium turned pink and there was an increase in number by day 7. The HNRB-P single culture microcosm required an increased inoculum (1.2%) in order to grow in the CSB(2) medium. This isolate turned the medium slightly yellow before turning pink by day 10 and sulfide was not removed from the culture until day 10 (Table 6.1).

For the NR-SOB enrichment cultures, sulfide was removed, NO_2^- was formed and the medium turned pink (Table 6.1). The fact that there did not appear to be any change in NO_3^- concentrations for the NR-SOB enrichment cultures was likely due to the fact that the CSB(2) medium contained high NO_3^- and low sulfide concentrations. The detection method for NO_3^- was not sensitive enough to show small changes in the NO_3^- concentration. The NR-SOB MPN results showed an increase in number for NR-SOB-C by day 7 and a slow increase for NR-SOB-N by day 21. The NR-SOB-P number on day 7 was the same as day 0 and then decreased dramatically by day 14. The NR-SOB-P appeared to be active in the microcosm because sulfide was removed and NO_2^- increased (Table 6.1). From the chemical analyses, it was expected that the NR-SOB-P numbers should have increased over the testing period. There are two possible explanations for the NR-SOB-P MPN results. First, the NR-SOB-P did not respond favorably to the MPN procedure. Second, the NR-SOB-P enrichment culture did not survive after day 7 in the microcosm. On day 3, sulfide was depleted and NO_2^- was 0.8 mM. The NO_2^- concentration did not change after day 3 and the culture decreased in number after day 7.

Table 6.1 Preliminary single culture microcosm studies for HNRB and NR-SOB from oil fields P, N and C

Oil field cultures	Parameters monitored[a]					Microcosm changes[a]	
HNRB isolates	Inoculum used in the microcosms	Sulfide[b]	Acetate[b]	NO_3^{-b}	NO_2^{-b}	MPN mL^{-1}	Color in medium
		100% loss by:	Decrease by day 7		Increase by day 7		
HNRB-P	1.2%[c]	Day 10	50 %	35 %	1.4 mM	22-fold increase[d]	Slightly yellow, days 2 and 3; Pink by day 10
HNRB-N	0.6%	Day 3	100 %	75 %	4.3 mM	10-fold increase[d]	Pink by day 2
HNRB-C	0.6%	Day 3	100 %	80 %	6.6 mM	10-fold increase[d]	Pink by day 2
NR-SOB enrichment cultures							
NR-SOB-P	3%	Day 3	None	None	0.8 mM	4000-fold decrease[e]	Pink by day 2
NR-SOB-N	3%	Day 3	None	None	0.6 mM	15-fold increase[f]	Pink by day 3
NR-SOB-C	3%	Day 3	None	None	2.2 mM	53-fold increase[g]	Pink by day 1

[a] Chemical analyses were done on days 0, 3, 7, 10, 14 and 21 and MPN procedures were done on days 0, 7, 14 and 21

[b] Initial concentrations in medium: Sulfide, 0.8 mM ; $SO_4^{=}$, 20 mM; Acetate, 10 mM; Benzoate, 1.8 mM; NO_3^-, 20 mM; NO_2^-, below detection

[c] The microcosm with 0.6% inoculum did not grow and 1.2% inoculum was used.

[d] Change in MPN value by day 7.

[e] MPN result, day 7 was the same as day 0 and then decreased by day 14.

[f] MPN result, day 7 was the same as day 0, day 14 MPN index was not probable and the increase was shown by day 21.

[g] MPN result, day 7 showed a 22-fold increase and day 14 showed a 53-fold increase from day 0.

The results for NR-SOB-P and -N were consistent with results obtained during the enrichment work for these bacteria.

After both sets of microcosm studies were completed, the microcosms were analyzed for N_2O. There was N_2O detected in all HNRB, NR-SOB-N and NR-SOB-C microcosms but no N_2O detected in the sterile medium control or the NR-SOB-P microcosms. These microcosms did not have acetylene added to block the reduction of N_2O. For each set of oil field cultures, with the exception of HNRB-P, the HNRB and NR-SOB turned the medium pink at about the same time and the HNRB produced more NO_2^- than the NR-SOB (Table 6.1).

6.3.4 *Final studies with single, double and triple culture microcosms for oil field water C, P and N*

6.3.4.1 Oil field C

For the oil field C microcosm studies, Table 6.2 shows the microcosm changes, Figure 6.1 shows the chemical changes and Table 6.3 shows the acetate and benzoate changes in the microcosms. The results for the sterile medium control microcosm showed no change in sulfide, $SO_4^=$, NO_3^-, NO_2^-, N_2O, acetate or benzoate over the testing period.

The results for oil field C single culture microcosm studies are as follows. In these microcosm studies, the HNRB, NR-SOB and SRB were incubated in CSB(2) medium alone. The HNRB-C did not appear to grow or produce changes in the single culture microcosm (Tables 6.2, 6.3; Figure 6.1e). There was a 1000-fold decrease in number by day 7. In the preliminary microcosm studies (section 6.3.3, Table 6.1), the HNRB-C removed sulfide by day 3 and grew in the CSB(2) medium using acetate and NO_3^-.

In the NR-SOB-C single culture microcosm, sulfide was removed (Figure 6.1c), the microcosm turned pink by day 1 and NO_2^- was detected from days 1 to 14 (Figure 6.1c, Table 6.2). Nitrous oxide was detected in the microcosm with acetylene on days 1 and 14 (Table 6.2). The NR-SOB-C MPN result increased 100-fold by day 7. In the preliminary microcosm studies (section 6.3.3, Table 6.1), the NR-SOB-C also removed sulfide, produced NO_2^-, turned the medium pink and produced N_2O.

Table 6.2 Microcosm changes in single, double, and triple culture microcosm studies for oil field C

	Single culture microcosms			Double culture microcosms			Triple culture microcosm
Parameters monitored	HNRB-C	NR-SOB-C	SRB[a]	HNRB-C; NR-SOB-C	NR-SOB-C; SRB[a]	HNRB-C; SRB[a]	HNRB-C; NR-SOB-C; SRB[a]
Color change	No change	Pink	No change	Pink[b]	Pink	No change	Pink[c]
Day changed		1		1	1		1
Turbidity	Not turbid	Not turbid	Turbid	Turbid	Turbid	Turbid	Turbid
Day changed			4	7	7	7	7
NO_2^- detected[d]	None	Detected	None	Detected	Detected	None	Detected
Days detected		1 to 14		1	1 to 14		1
N_2O detected[e]	0.05%[f]	1%	None	2 to 4%	3%	0.05%[g]	3 to 4%
Days detected	7, 14	1, 14		1 to 14	1, 14	7, 14	1 to 14

[a] SRB was *D. multivorans*

[b] Day 4, slightly pink; day 7, colorless; day 14, only pink after shaking

[c] Colorless from days 2 to 14

[d] NO_2^- was analyzed on days 1, 2, 4, 7, 10 and 14.

[e] N_2O (% v v^{-1}) was detected in a separate microcosm with acetylene added. N_2O was analyzed on: days 1, 14 for NR-SOB-C, NR-SOB-C plus SRB; days 7, 14 for HNRB-C, SRB, HNRB-C plus SRB; days 1, 4, 7, 14 for HNRB-C plus NR-SOB-C, triple culture.

[f] The small amount of N_2O was likely carryover from the inoculum

[g] There was more N_2O detected in this microcosm than in the single culture HNRB-C microcosm

Table 6.3 Decrease in acetate and benzoate concentrations in microcosm studies containing CSB(2) medium and single, double and triple cultures for oil field C

	Single culture microcosm			Double culture microcosm			Triple culture microcosm
Parameters monitored[a]	HNRB-C	NR-SOB-C	SRB[b]	HNRB-C; NR-SOB-C	HNRB-C; SRB[b]	NR-SOB-C; SRB[b]	HNRB-C; NR-SOB-C; SRB[b]
Acetate[c]	No change	No change	32%, day 14	12%, day 14	14%, day 14	No change	46%, day 14
Benzoate[c]	No change	No change	65%, day 14	No change	78%, day 14	No change	48%, day 14

[a] The microcosms were analyzed on days 0, 1, 2, 4, 7, 10 and 14.

[b] SRB was *D. multivorans*

[c] Results showing acetate (15 mM) and benzoate (1 mM) decrease by the day indicated

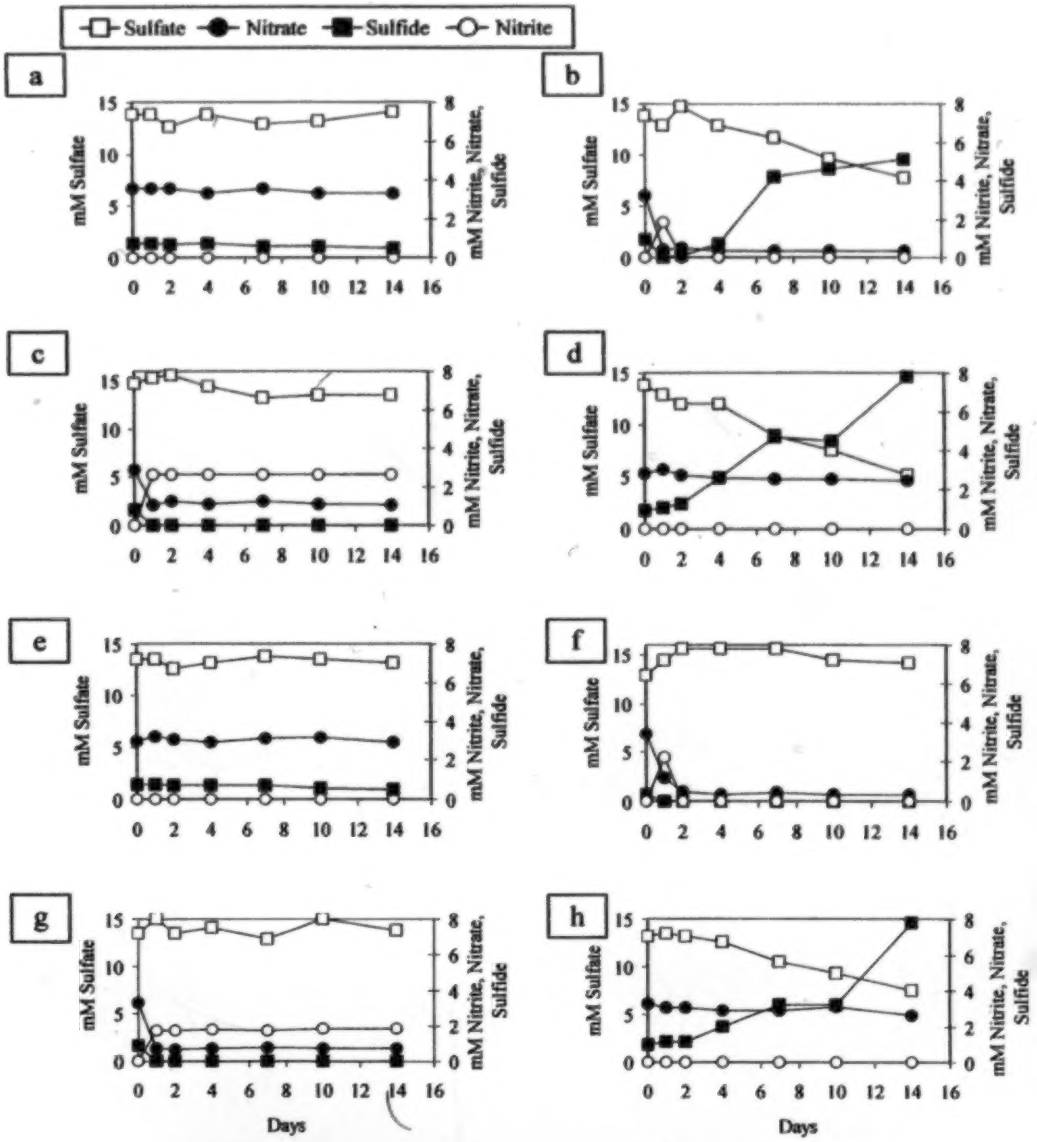

Figure 6.1 Chemical changes for NO_3^-, NO_2^-, $SO_4^=$ and sulfide in microcosms with cultures for oil field C. Initial concentrations in CSB(2) medium: NO_3^-, 3 mM; NO_2^-, below detection; $SO_4^=$, 15 mM; Sulfide, 0.8 mM

(a) medium control
(b) three cultures, HNRB-C, NR-SOB-C and *D. multivorans*
(c) one culture NR-SOB-C
(d) one culture, *D. multivorans*
(e) one culture, HNRB-C
(f) two cultures, HNRB-C and NR-SOB-C
(g) two cultures, *D. multivorans* and NR-SOB-C
(h) two cultures, HNRB-C and *D. multivorans*

In 14 d, the SRB single culture microcosm increased the sulfide concentration from 0.9 mM to 8 mM and used $SO_4^=$ (Figure 6.1d). Benzoate and acetate were also used (Table 6.3). The SRB MPN result increased 13-fold by day 7.

The results for oil field C double culture microcosm studies are as follows. In these microcosm studies, two cultures of HNRB, NR-SOB or SRB were combined and incubated in CSB(2) medium. In the microcosm with the two cultures, HNRB-C plus NR-SOB-C, the sulfide was removed (Figure 6.1f) and the medium in the microcosm became pink (Table 6.2). Nitrite was detected only on day 1 (Table 6.2, Figure 6.1f). The medium in the microcosm became colorless and turbid by day 7 and on day 14 was pink only when shaken, indicating that some N_2O was dissolved in the medium. A large amount of N_2O was detected from days 1 to 14 in the microcosm with acetylene (Table 6.2). There was more N_2O detected in this microcosm than in the single culture microcosms with HNRB-C and NR-SOB-C. Acetate was slowly used over the 14 d (Table 6.3). The HNRB-C MPN result decreased 22-fold by day 7 and then increased 100-fold by day 14. The NR-SOB-C MPN result increased 100-fold by day 7.

In the microcosm with the two cultures, HNRB-C plus SRB, sulfide increased from 0.9 mM to 8 mM (Figure 6.1h). There was a decrease in $SO_4^=$ and benzoate (Figure 6.1h, Table 6.3). Acetate decreased slightly from days 10 to 14 (Table 6.3). A small increase in N_2O was detected on days 7 and 14 in the microcosm with acetylene (Table 6.2). The HNRB-C MPN result decreased from 10^6 on day 0 to 10^1 by day 7 and then increased 1000-fold from day 7 to day 14. The SRB MPN result increased 1000-fold by day 7 and no MPN index could be determined for day 14. There is no clear evidence from the microcosm chemical analyses to explain the HNRB-C MPN increase on day 14. There was no obvious decrease of NO_3^- and increase of NO_2^- (Figure 6.1h, Table 6.2). On day 14, in the microcosms with acetylene, there was slightly more N_2O detected in the HNRB-C plus SRB microcosm than in the single culture microcosm HNRB-C (Table 6.2). The HNRB-C MPN results from other mixed microcosms initially decreased on day 7 before increasing on day 14.

In the microcosm with the two cultures, NR-SOB-C plus SRB, by day 1 sulfide was removed (Figure 6.1g) and the medium in the microcosm turned pink (Table 6.2). In this microcosm, NO_2^- was detected from days 1 to 14 (Table 6.2). Because the NR-SOB-

C could produce N_2O (Table 6.2), it is possible that NO_2^- and N_2O were present in the microcosm. Nitrous oxide was detected on days 1 and 14 in the microcosm with acetylene (Table 6.2) and more N_2O was detected in this microcosm than when NR-SOB-C was incubated alone. There was no change in $SO_4^=$, acetate or benzoate (Table 6.3, Figure 6.1g). The NR-SOB-C MPN result increased 50-fold by day 7. No MPN index was obtained for the SRB on day 0 but the results for days 7 and 14 were the same at 10^6.

The results for the oil field C triple culture microcosm study are as follows. In this microcosm study, HNRB, NR-SOB and SRB were combined and incubated in CSB(2) medium. Sulfide was removed, the medium in the microcosm turned pink by day 1 and NO_2^- was detected only on day 1 (Figure 6.1b). A large amount of N_2O was detected from days 1 to 14 in the microcosm with acetylene (Table 6.2). The $SO_4^=$, acetate and benzoate concentrations showed an obvious decrease after day 4 (Table 6.3, Figure 6.1b). The sulfide concentration increased to 5 mM from days 2 to 14 and the medium in the microcosm became colorless (Figure 6.1b, Table 6.2). The medium in the microcosm for N_2O detection turned pink when shaken and no sulfide analyses were done on this microcosm. For the MPN results, the HNRB-C number decreased 25-fold from day 0 to day 7 and then increased 4000-fold from day 7 to day 14. The NR-SOB-C number increased 100-fold by day 7 and the SRB number showed no change even though the sulfide concentration increased. On day 14, 10 mM NO_3^- was added to the three microcosms. Within 12 h, the medium in all the microcosms became pink and the sulfide was removed.

For the triple culture microcosm, examination of the chemical analyses did not provide evidence that the HNRB-C was active because the results were confounded by the presence of the NR-SOB-C and SRB. In this microcosm, the NR-SOB-C could have reduced the NO_3^- (Figure 6.1c) and the SRB could have used the acetate (Table 6.3). One indication that the HNRB-C was active was from the MPN result. There was a significant decrease in number from day 0 to day 7 and then a significant increase in number from day 7 to day 14. The validity of these MPN results could be questionable because the sulfide concentration in the microcosm was 4 mM on Day 7. When HNRB-C was incubated alone during the enrichment process (section 6.3.1), it was inhibited by sulfide concentrations >0.8 mM. If however, the HNRB-C was not active in the microcosm, the chemical analyses shown in Figure 6.1b for the triple culture microcosm should have been the same as for the double culture microcosm with the NR-SOB-C plus SRB, shown in Figure 6.1g. This was not the case.

In the triple culture microcosm, on day 1, sulfide was below detection, NO_3^- decreased to 0.4 mM and the NO_2^- concentration was 1.8 mM. The HNRB-C could have been active in this microcosm because the NO_3^- concentration decreased 2.8 mM. This decrease in NO_3^- is more than the 1.3 mM stoichiometric requirement for sulfide oxidation by NR-SOB (assuming 0.8 mM sulfide is converted to $SO_4^{=}$ and NO_3^- to N_2, as shown in equation 6.1) and more than the 1.8 mM decrease when NR-SOB-C was

$$3H^+ + 8NO_3^- + 5HS^- \rightarrow 4N_2 + 5SO_4^{=} + 4H_2O \qquad (6.1)$$

incubated alone (Figure 6.1c). There was a large amount of N_2O detected in the triple culture microcosm with acetylene, and it was assumed that N_2O was produced in the triple culture microcosm with no acetylene (Table 6.2). By day 2, the sulfide concentration was 0.1 mM, the NO_3^- concentration remained at 0.4 mM and the NO_2^- concentration was below detection. By day 4, the sulfide concentration was 0.7 mM, the NO_3^- concentration was 0.3 mM and the NO_2^- concentration remained below detection. It appears that in this microcosm, after day 2, the nitrate-reducing activity declined. The NO_3^- concentration had decreased 90%, NO_2^- and possibly N_2O were depleted and the SRB became active. There was no clear indication as to whether the HNRB-C or NR-SOB-C removed the NO_2^- and possibly N_2O after day 1. The HNRB-C could have removed the NO_2^- because it can use acetate as an energy source. If the NR-SOB-C reduced the NO_2^-, then the SRB would have had to produce the sulfide needed by NR-SOB-C for an energy source. In other microcosms with NR-SOB-C, NO_2^- accumulated when sulfide was depleted (Figures 6.1c, 6.1g). For the triple culture microcosm, there was 0.1 mM sulfide in the microcosm on day 2 and by day 14, the sulfide concentration was 5 mM (Figure 6.1b). When NO_3^- was added to the triple microcosms on day 14, nitrate-reduction activity appeared to resume because sulfide was removed. It was assumed that the NR-SOB-C removed the sulfide in the microcosms because NR-SOB that tolerate up to 15 mM sulfide have been characterized from oil field C (Telang et al.

1999). The HNRB-C, used in this study, did not tolerate sulfide concentrations >0.8 mM when isolated alone.

In summary, for oil field C, the HNRB-C did not actively grow when incubated in the CSB(2) medium alone (Figure 6.1e; Tables 6.2, 6.3). In all cases, when incubated in the presence of the NR-SOB-C (Figure 6.1f) and, or SRB (Figures 6.1b, 6.1h; respectively), the HNRB-C number significantly decreased by day 7 and then increased by day 14. It appears as though the benzoate and, or sulfide may have initially inhibited the HNRB-C when it was transferred to the microcosms. The removal of sulfide by the NR-SOB-C or benzoate by the SRB may have relieved the inhibition to the HNRB-C in the microcosms. The day 14 MPN increase for HNRB-C in the presence of SRB is perplexing because the NO_3^- and NO_2^- concentrations did not appear to change.

Whenever the HNRB-C was incubated in the presence of the NR-SOB-C, there was a transient increase in NO_2^- (Figures 6.1b, 6.1f) and a large amount of N_2O was produced (Table 6.2). The NR-SOB-C quickly removed sulfide when incubated alone and in the double culture microcosms with HNRB-C and SRB (Figures 6.1c, 6.1f, 6.1g).

The SRB appeared to be temporarily inhibited in the triple culture microcosm when NO_3^- was not limiting and NO_2^- was present (Figure 6.1b, Table 6.2). It produced sulfide after day 1 but did not increase in number in the triple culture microcosm. The SRB did not produce sulfide when incubated in the double culture microcosm with the NR-SOB-C (Figure 6.1g). The SRB did produce sulfide in the double culture microcosm with the HNRB-C (Figure 6.1h) and when NO_2^- was below detection in the triple culture microcosm (Figure 6.1b).

6.3.4.2 Oil field P

For the oil field P microcosm studies, Table 6.4 shows the microcosm changes, Figure 6.2 shows the chemical changes and Table 6.5 shows the acetate and benzoate changes in the microcosms. The results for the sterile medium control microcosm showed no change in sulfide, $SO_4^{=}$, NO_3^-, NO_2^-, N_2O, acetate and benzoate over the testing period.

Table 6.4 Microcosm changes in single, double and triple culture microcosm studies for oil field P

	Single culture microcosms			Double culture microcosms			Triple culture microcosm
Parameters monitored	HNRB-P[a]	NR-SOB-P	SRB[b]	HNRB-P; NR-SOB-P	NR-SOB-P; SRB[b]	HNRB-P; SRB[b]	HNRB-P; NR-SOB-P; SRB[b]
Color change	Pink[c]	Pink	Colorless	Pink[d]	Pink	Yellow	Yellow
Day changed	10	1		3	1	3	3
Turbidity	Turbid	Turbid[e]	Turbid	Turbid	Turbid[f]	Turbid	Turbid[g]
Day changed	3	1	1	3	1	1	1
NO_2^- detected[h]	Detected	Detected	None	Detected	Detected	None	None
Days detected	3, 5, 7	1 to 14		3	1 to 14		
N_2O detected[i]	0.05 to 1%	None	None	1 to 4%	None	0.05 to 1%	0.05 to 1%
Days detected	1 to 14			1 to 14		1 to 14	1 to 14

[a] HNRB-P was clumped and adhered to the bottom of the serum bottle in all microcosms

[b] SRB was *D. multivorans*

[c] The color was slightly pink after shaking

[d] Day 3, slight pink; day 7, pink; day 14, pink only when shaken

[e] Turbidity appeared to be a precipitate

[f] Turbid days 1 to 7 and a precipitate day 10

[g] Microcosm was not turbid after day 7

[h] NO_2^- was analyzed on days 1, 3, 5, 7, 10 and 14

[i] N_2O (% v v^{-1}) was detected in a separate microcosm with acetylene added. N_2O was analyzed on days 1, 3, 7 and 14.

Table 6.5 Decrease in acetate and benzoate concentrations in microcosm studies containing CSB(2) medium and single, double and triple culture microcosms for oil field P

	Single culture microcosms			Double culture microcosms			Triple culture microcosm
Parameters monitored[a]	HNRB-P	NR-SOB-P	SRB[b]	HNRB-P; NR-SOB-P	HNRB-P; SRB[b]	NR-SOB-P; SRB[b]	HNRB-P; NR-SOB-P; SRB[b]
Acetate[c]	27%, day 14	No change	53%, day 14	24%, day 14	No change	No change	No change
Benzoate[c]	No change	No change	100%, day 10	No change	No change	No change	No change

[a] Microcosms were analyzed on days 1, 3, 5, 7, 10 and 14
[b] SRB was *D. multivorans*
[c] Results showing acetate (25 mM) and benzoate (1 mM) decrease by the day indicated.

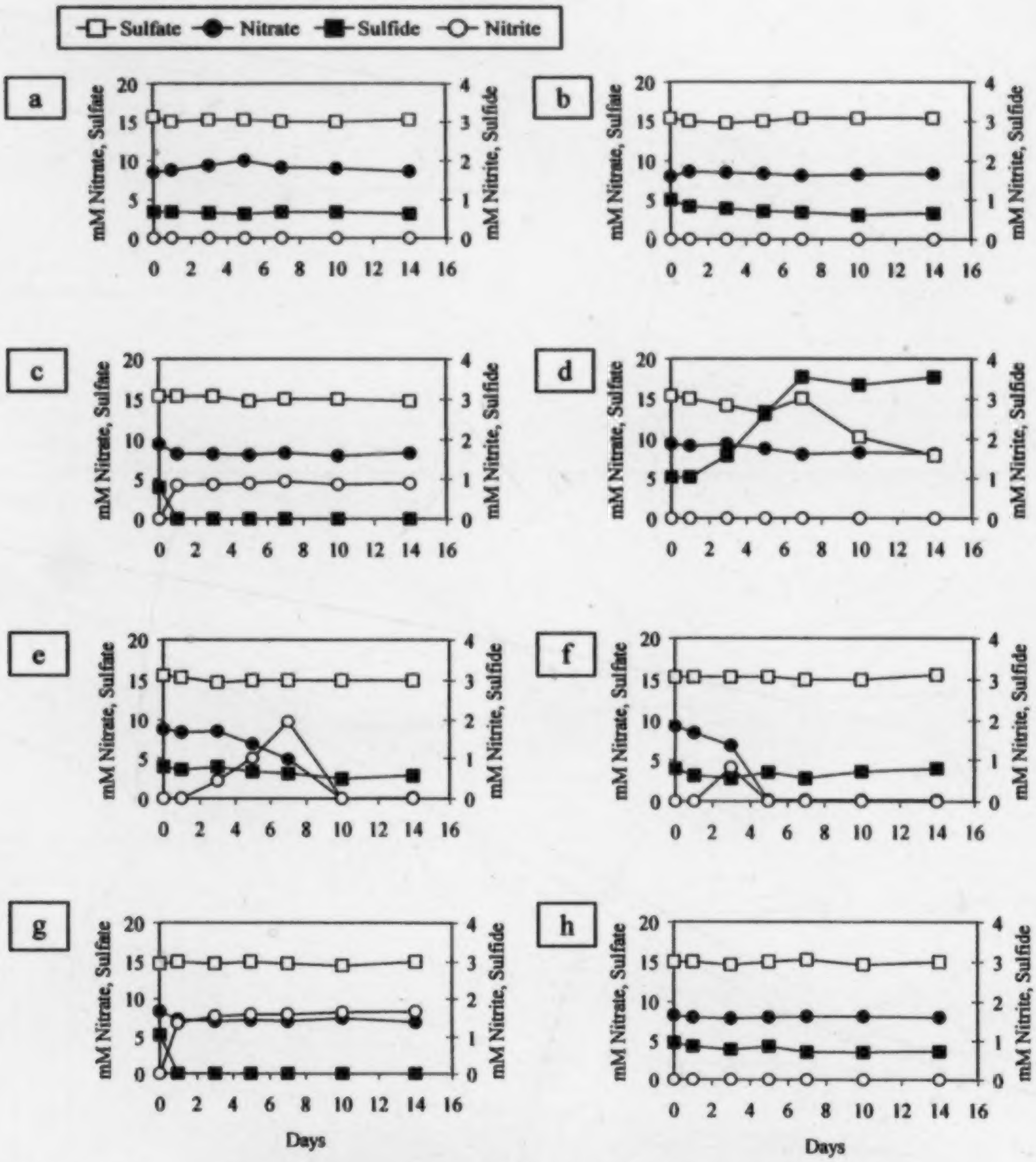

Figure 6.2 Chemical changes for NO_3^-, NO_2^-, $SO_4^=$ and sulfide in microcosms with cultures for oil field P. Initial concentrations in CSB(2) medium: NO_3^-, 10 mM; NO_2^-, below detection; $SO_4^=$, 15 mM; Sulfide, 0.8 mM
(a) medium control
(b) three cultures, HNRB-P, NR-SOB-P and *D. multivorans*
(c) one culture, NR-SOB-P
(d) one culture, *D. multivorans*
(e) one culture, HNRB-P
(f) two cultures, HNRB-P and NR-SOB-P
(g) two cultures, *D. multivorans* and NR-SOB-P
(h) two cultures, HNRB-P and *D. multivorans*

The HNRB-P adhered to the bottom of the serum bottles making it difficult to remove and mix thoroughly for MPN procedures. As a result, all the serum bottles that were used in the MPN procedures (SB1) were sonicated in a water bath for 5 min on day 7. MPN procedures (1-tube) were done for each microcosm set on day 14 using the unsonicated second serum bottle (SB2). These MPN tests were done to determine if the microcosms (SB1) were affected by sonication.

The results for oil field P single culture microcosm studies are as follows. In these microcosm studies, the HNRB, NR-SOB and SRB were incubated in CSB(2) medium alone. For all the single culture microcosms, the day 14 MPN results obtained for SB2 were the same as SB1.

The HNRB-P removed NO_3^- by day 10 and the medium in the microcosm was slightly pink after shaking, indicating that N_2O may be dissolved in the medium. Sulfide was not removed (Figure 6.2e, Table 6.4). The HNRB-P decreased acetate, produced NO_2^- from days 3 to 7 and there was a 50-fold increase in number by day 7 (Tables 6.4, 6.5). There was an increasing amount of N_2O detected from days 1 to 14 in the microcosm with acetylene (Table 6.4). In the preliminary microcosm studies, the HNRB-P removed sulfide by day 10 and produce NO_2^- (section 6.3.3, Table 6.1) and N_2O.

In the NR-SOB-P single culture microcosm, sulfide was removed, the medium turned pink by day 1 and NO_2^- accumulated (Figure 6.2c, Table 6.4). Turbidity in the microcosms with the NR-SOB-P enrichment culture appeared as a precipitate (Table 6.4), which could have been elemental sulfur, as described by Jenneman et al. (1996). There was no N_2O detected at any time in the microcosm with acetylene (Table 6.4). The MPN result for the NR-SOB-P did not change from days 0 to 7 and decreased 200-fold by day 14. In the preliminary microcosm studies, the NR-SOB-P gave similar results (section 6.3.3, Table 6.1) and did not produce N_2O.

The SRB, in the single culture microcosm, increased the sulfide from 1 to 4 mM and decreased $SO_4^{=}$, acetate and benzoate (Figure 6.2d, Table 6.5). There was a 60-fold increase in number by day 7.

The results for oil field P double culture microcosm studies are as follows. In these microcosm studies, two cultures of HNRB, NR-SOB or SRB were combined and incubated in CSB(2) medium. In the microcosm with two cultures, HNRB-P plus NR-

SOB-P, the NO_3^- was removed by day 5, acetate decreased, NO_2^- was detected on day 3 only and sulfide was not removed (Figure 6.2f; Tables 6.4, 6.5). A large increasing amount of N_2O was detected from days 1 to 14 in the microcosm with acetylene, and the intensity of the pink color in the medium increased from days 3 to 7. By day 14, the medium was pink only after the microcosm was shaken and this was an indication that N_2O was dissolved in the medium (Table 6.4). For the HNRB-P, the MPN values showed a 25-fold increase by day 7 and a 15-fold decrease by day 14. For the NR-SOB-P, a MPN index was not obtained for day 7 and by day 14, there was a 500-fold decrease in number from day 0. The same day 14 MPN results were obtained for SB2.

In the microcosm with two cultures, HNRB-P plus SRB, there appeared to be no change although the medium in the microcosm turned yellow on day 3 (Figure 6.2h; Tables 6.4, 6.5). A small amount of N_2O was detected from days 1 to 14 in the microcosm with acetylene (Table 6.4). Although no MPN index could be obtained for HNRB-P on day 0, there appeared to be a decrease in number from about 10^5 on day 0 to 10^1 on days 7 and 14. The day 14 MPN result for SB2 was the same as SB1 for HNRB-P. The SRB MPN result in SB2 was lower than SB1. The SRB may have been clumping or may have adhered to the glass in the SB2 serum bottle.

In the microcosm with two cultures, NR-SOB-P plus SRB, sulfide was removed, the medium in the microcosm turned pink and NO_2^- accumulated (Figure 6.2g, Table 6.4). No N_2O was detected in the microcosm with acetylene (Table 6.4). For the NR-SOB-P, there was a 10-fold increase in number by day 7 and on day 14, the number was the same as day 0. For SRB, a MPN index was not obtained for day 7 and on day 14, there was no change in number from day 0. Comparing the day 14 MPN results for SB1 and SB2, the MPN result in SB2 was the same as SB1 for NR-SOB-P and the MPN result in SB2 was lower than SB1 for SRB. Again, the SRB MPN result in SB2 could indicate clumping or attachment of cells to the glass in the microcosm.

The results for oil field P triple culture microcosm study are as follows. In this microcosm study, HNRB, NR-SOB and SRB were combined and incubated in CSB(2) medium. For the HNRB-P and the NR-SOB-P, there was a decrease in numbers by day 7 of 100-fold and 40-fold, respectively, and for the SRB, there was a 15-fold increase in number by day 7. The medium in the microcosm was yellow and turbid from days 3 to 7 and then yellow and not turbid from days 10 to 14. It appeared as though sonication affected the triple culture microcosm (Figure 6.2b, Table 6.4). The two other triple culture microcosm serum bottles remained yellow and turbid to day 14. The day 14 MPN results for HNRB-P were higher in SB2 than SB1 and for NR-SOB-P, were lower in SB2 than SB1. No MPN index was obtained for the SRB in SB1 on day 14 so comparison could not be made to SB2.

In summary, for oil field P, the HNRB-P survived and grew in the single microcosm but did not remove sulfide (Figure 6.2e; Tables 6.4, 6.5). In the double culture microcosm, HNRB-P plus NR-SOB-P, the HNRB-P actively grew, produced large amounts of N_2O, did not remove sulfide and appeared to be more dominant than the NR-SOB-P in the microcosm (Figure 6.2f; Tables 6.4, 6.5). In this microcosm, the NR-SOB-P did not remove sulfide and decreased in number by day 14. The reason for the apparent dominance of HNRB-P in this microcosm was not obvious and could have been due to the characteristics of the microcosm or related to a kinetic advantage for the HNRB-P using acetate and NO_3^- in the microcosm. It was not likely related to a thermodynamic advantage, because the amount of energy gained by both NRB during nitrate reduction is about the same. The energy gained by NR-SOB per mol NO_3^- reduced using HS^- as an election donor (equation 3.3), is -491 kJ and the energy gained by HNRB per mol NO_3^- reduced using acetate as an electron donor (equation 4.2) is -495 kJ. The HNRB-P did not actively grow in the double culture microcosm with SRB (Figure 6.2h; Tables 6.4, 6.5) and did not actively grow in the first 7 d, before sonication, in the triple culture microcosm with SRB and NR-SOB-P (Figure 6.2b; Tables 6.4, 6.5). The HNRB-P appeared to be affected by sonication only in the triple culture microcosm.

The NR-SOB-P removed sulfide only when incubated alone and in the presence of SRB (Figures 6.2c, 6.2g). The NR-SOB-P did not produce N_2O (Table 6.4); produced NO_2^-, which accumulated (Figures 6.2c, 6.2g); inhibited SRB (Figure 6.2g); increased in number only in the presence of SRB and did not appear to be affected by sonication.

The SRB did not produce sulfide in the presence of NR-SOB-P (Figure 6.2g) and, or HNRB-P (Figures 6.2b, 6.2h). The sulfide was increased when the SRB was incubated alone (Figure 6.2d).

6.3.4.3 Oil field N

For the oil field N microcosm studies, Table 6.6 shows the microcosm changes, Figure 6.3 shows the chemical changes and Table 6.7 shows the acetate and benzoate changes in the microcosms. The results for the sterile medium control microcosm showed no change in sulfide, $SO_4^{=}$, NO_3^-, NO_2^-, N_2O, acetate and benzoate over the testing period.

The results for oil field N single culture microcosm studies are as follows. In these microcosm studies, the HNRB, NR-SOB and SRB were incubated in CSB(2) medium alone. The HNRB-N did not appear to grow in any of the microcosms (Figures 6.3b, 6.3e, 6.3f, 6.3h; Tables 6.6, 6.7). When incubated alone in the single culture microcosm, the medium in the microcosm turned yellow by day 3 and a stringy substance appeared. The substance dispersed when the microcosm was shaken (Table 6.6). MPN results for all microcosms containing HNRB-N showed growth only on day 0. The HNRB-N grew well in the CSB(2) medium during the preliminary microcosm studies, removed sulfide by day 3 and used acetate and NO_3^-. The results are given in section 6.3.3 and Table 6.1.

In the NR-SOB-N single culture microcosm, the 0.8 mM sulfide decreased 80% by day 14 and NO_2^- accumulated (Figure 6.3c). The medium in the microcosm turned yellow by day 3 and surprisingly, no N_2O was detected in the microcosm with acetylene (Table 6.6). The NR-SOB-N MPN value increased 50-fold by day 7. In the preliminary microcosm studies, the NR-SOB-N reduced NO_3^-, removed sulfide, turned the CSB(2) medium pink by day 3 (section 6.3.3, Table 6.1) and produced N_2O.

In the single culture microcosm with SRB, sulfide increased from 1 to 3 mM and $SO_4^{=}$ and benzoate decreased (Figure 6.3d, Table 6.7). There was no obvious change in the acetate concentration for any of the microcosms with the SRB (Table 6.7). There was no MPN index for the SRB on day 7 but there was an increase in number of 100-fold from days 0 to 14.

Table 6.6 Microcosm changes in single, double and triple culture microcosm studies for oil field N

	Single culture microcosms			Double culture microcosms			Triple culture microcosm
Parameters monitored	HNRB-N[a]	NR-SOB-N	SRB[b]	HNRB-N; NR-SOB-N	NR-SOB-N; SRB[b]	HNRB-N; SRB[b]	HNRB-N; NR-SOB-N; SRB[b]
Color change	Yellow	Yellow	Colorless	Yellow, day 3;	Pink[d]	Yellow	Yellow, day 3;
Day changed	3	3		Pink, day 28[c]	7	3	Pink, day 28[c]
Turbidity	Stringy[e]	Flocculent[e]	Turbid	Turbid	Turbid	Turbid	Turbid
Day changed	5	7	3	3	3	3	3
NO_2^- detected[f]	None	Detected	None	None	Detected	None	None
Days detected		3 to 14			3 to 14		
N_2O detected[g]	0.05%[h]	None	None	None	None	0.05 to 1%	0.05%[h,i]
Days detected	3 to 14					3 to 14	3

[a] HNRB-N had a stringy appearance that dispersed when shaken. The isolate did not appear to be alive.

[b] SRB was *D. multivorans*

[c] The microcosms were observed for changes in color from days 14 to 28 and analyzed for sulfide on day 28. The sulfide was removed by day 28.

[d] Day 3, yellow

[e] At the bottom of the microcosm

[f] NO_2^- was analyzed on days 1, 3, 5, 7, 10 and 14

[g] N_2O (% v v^{-1}) was detected in a separate microcosm with acetylene added. N_2O was analyzed on days 3, 5, 7 and 14.

[h] The small amount of N_2O was likely carryover from the inoculum

[i] Detected only on day 3

Table 6.7 Decrease in acetate and benzoate concentrations in microcosm studies with CSB(2) medium and single, double and triple culture microcosms for oil field N

	Single culture microcosms			Double culture microcosms			Triple culture microcosm
Parameters monitored[a]	HNRB-N	NR-SOB-N	SRB[b]	HNRB-N; NR-SOB-N	HNRB-N; SRB[b]	NR-SOB-N; SRB[b]	HNRB-N; NR-SOB-N; SRB[b]
Acetate[c]	No change	No change	No change	No change	No change	No change	No change
Benzoate[c]	No change	No change	73%, day 14	No change	No change	No change	No change

[a] Microcosms were analysed on days 1, 3, 5, 7, 10 and 14.
[b] SRB was *D. multivorans*
[c] Results showing acetate (25 mM) and benzoate (1 mM) decrease by the day indicated.

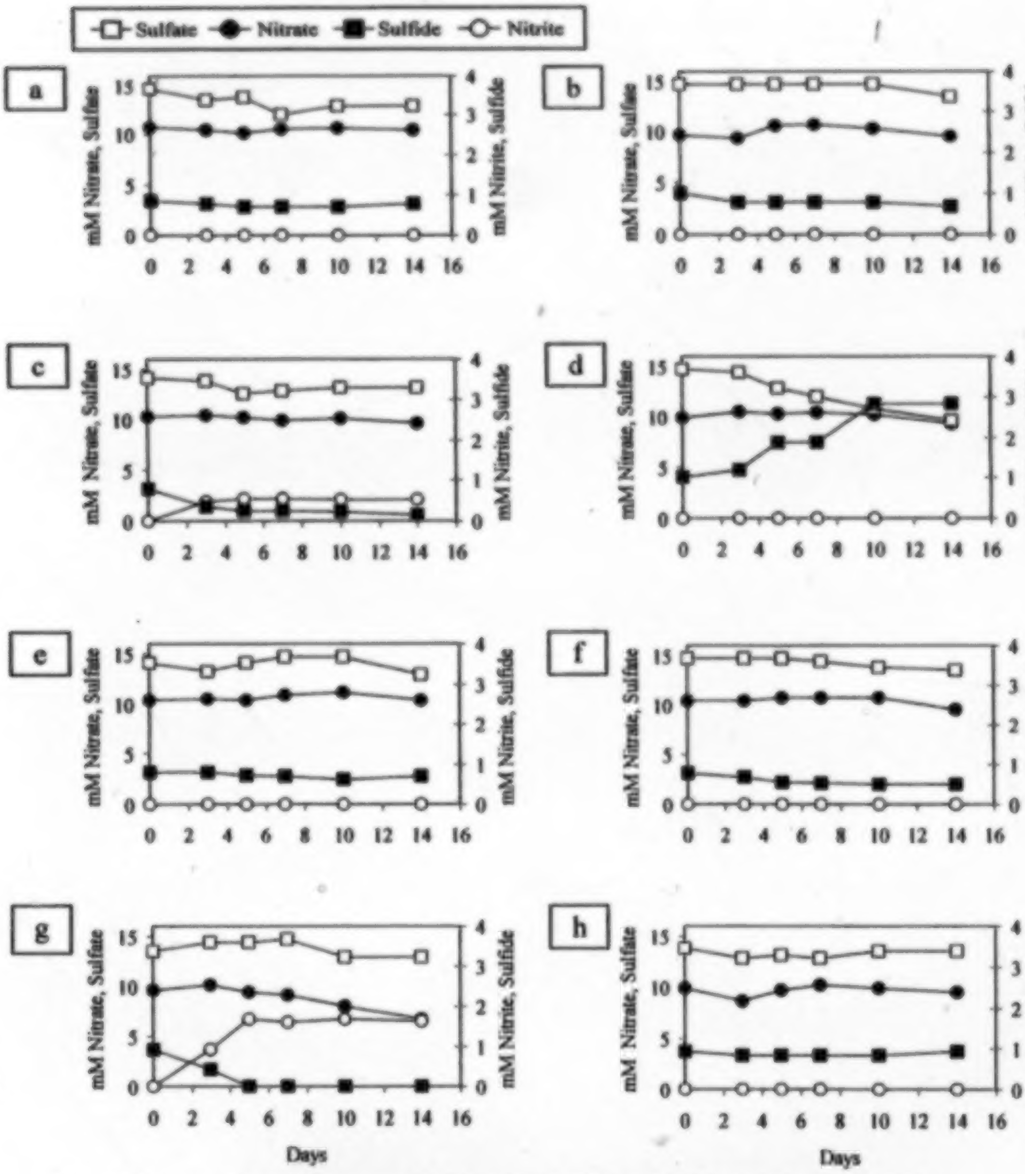

Figure 6.3 Chemical changes for NO_3^-, NO_2^-, $SO_4^=$ and sulfide in microcosms with cultures for oil field N. Initial concentrations in CSB(2) medium: NO_3^-, 10 mM; NO_2^-, below detection; $SO_4^=$, 15 mM; Sulfide, 0.8 mM

(a) medium control
(b) three cultures, HNRB-N, NR-SOB-N and *D. multivorans*
(c) one culture, NR-SOB-N
(d) one culture, *D. multivorans*
(e) one culture, HNRB-N
(f) two cultures, HNRB-N and NR-SOB-N
(g) two cultures, *D. multivorans* and NR-SOB-N
(h) two cultures, HNRB-N and *D. multivorans*

The results for oil field N double culture microcosm studies are as follows. In these microcosm studies, two cultures of HNRB, NR-SOB or SRB were combined and incubated in CSB(2) medium. In the microcosm with two cultures, HNRB-N plus NR-SOB-N, the 0.8 mM sulfide was decreased 38% by day 14 although there did not appear to be any change in the NO_3^- and NO_2^- concentrations over the 14 d (Figure 6.3f). The medium in the microcosm was yellow on day 3 (Table 6.6). The HNRB-N was not viable by day 7, and the NR-SOB-N MPN value increased 20-fold by day 7. There was no N_2O detected in the microcosm with acetylene (Table 6.6). The microcosms were examined for color change and sulfide loss after day 14. By day 28, the medium for the HNRB-N plus NR-SOB-N microcosms was pink, turbid and contained no sulfide (Table 6.6). No other chemical analyses or MPN procedures were done on day 28 for the microcosms. The NR-SOB-N enrichment culture likely produced the changes seen on day 28 in the microcosms, because the HNRB-N did not appear to be alive on day 14.

In the microcosm with two cultures, HNRB-N plus SRB, there appeared to be no chemical changes (Figure 6.3h; Tables 6.6, 6.7). The medium in the microcosm was yellow on day 3. The HNRB-N was not viable by day 7. There was no change in the MPN value for the SRB, indicating that there may have been something in the microcosm that was toxic to the SRB.

In the microcosm with two cultures, NR-SOB-N plus SRB, sulfide was removed by day 5, NO_2^- accumulated and the medium in the microcosm was pink by day 7 (Figure 6.3g, Table 6.6). For the MPN enumerations, the NR-SOB-N number increased 90-fold by day 7 and there was no change in the SRB number. There was no N_2O detected in the microcosm with acetylene (Table 6.6).

The results for oil field N triple culture microcosm study are as follows. In this microcosm study, HNRB, NR-SOB and SRB were combined and incubated in CSB(2) medium. The microcosm showed no change except the medium turned a yellow color on day 3 (Figure 6.3b, Table 6.6). A scant amount of N_2O was detected only on day 3 in the microcosm with acetylene (Table 6.6). The HNRB-N was not viable by day 7. The MPN results showed no change in number for the SRB and the NR-SOB-N showed a 30-fold increase in number on day 14. The triple culture microcosms were examined for color change and sulfide loss after day 14. By day 28, the medium in the microcosms was pink, turbid and contained no sulfide (Table 6.6). No other chemical analyses or MPN procedures were done on day 28 for the microcosms. Again, the NR-SOB-N enrichment culture likely produced the changes that were seen on day 28 because the HNRB-N did not appear to be alive on day 14.

In summary, the HNRB-N did not survive after being inoculated to the microcosms with CSB(2) medium (Figures 6.3b, 6.3e, 6.3f, 6.3h; Tables 6.6, 6.7). It is likely that the HNRB-N was inhibited by the combination of sulfide and benzoate in the CSB(2) medium.

The SRB produced sulfide when incubated alone (Figure 6.3d) but did not produce sulfide when combined with the NR-SOB-N (Figure 6.3g). This could be due to inhibition of SRB by the presence of NO_2^- in this microcosm. The SRB did not produce sulfide when incubated with the HNRB-N (Figure 6.3h). There is no clear explanation for this because the HNRB-N appeared to be inactive in the microcosm. The SRB did not appear to use acetate in any of the microcosms (Table 6.7).

Activity in the triple culture microcosm was slow or delayed and no obvious changes were seen until after day 14. The NR-SOB-N quickly removed sulfide when incubated with the SRB (Figure 6.3g) and took more than 14 days to remove sulfide when incubated with HNRB-C and in the triple culture microcosm (Figures 6.3f and 6.3b, respectively; Table 6.6). The NR-SOB-N did not produce N_2O (Table 6.6) as it did in the preliminary studies (section 6.3.3).

6.4 Discussion

Soon after the this study began, it became clear that the oil field HNRB were fastidious and that each oil field has its own particular chemolithotrophic NR-SOB. The results from the preliminary microcosm studies (section 6.3.3) showed that both the oil field NR-SOB enrichment cultures and the HNRB isolates, when incubated alone, removed sulfide, reduced NO_3^- and produced the intermediate products NO_2^- and, or N_2O (Table 6.1). The final study (section 6.3.4) was used to observe changes in laboratory microcosms during incubations of various combinations of oil field HNRB isolates, oil field chemolithotrophic NR-SOB enrichment cultures and SRB in mineral medium with sulfide, $SO_4^=$, NO_3^- and two carbon sources. The carbon sources were not to be shared by the HNRB and SRB in order to avoid competition for a carbon source. Acetate was to be used by HNRB and benzoate by heterotrophic SRB. In their review, Magot et al. (2000) mention that short chain organic acids are present in petroleum reservoirs with acetate being the most abundant and benzoate, butyrate, formate and propionate commonly detected. Other studies have used acetate to isolate or enrich for heterotrophic oil field bacteria (Sperl et al. 1993, Reinsel et al. 1996, Wright et al. 1997, Larsen 2002, Thorstenson et al. 2002). Thorstenson et al. (2002) used benzoate to enrich for SRB in their study for NO_3^- treatment to injection water on a North Sea oil platform. These studies are from oil reservoirs that vary in depth and temperature with many having temperatures above 30°C. For the studies described in this thesis, the oil fields were shallow and mesophilic (Chapters 3, 4 and Eckford and Fedorak 2002b, 2002c) and their organic acids were not characterized. Acetate and benzoate were chosen as carbon sources for the final study because the HNRB from oil fields C, N and P all used acetate and appeared to be able to tolerate low concentrations of benzoate.

During the final microcosm studies (section 6.3.4), six problems became evident. The first problem was that none of the oil field HNRB isolates performed as expected. The HNRB were easily grown in CSB(2) medium and enumerated with nutrient broth-nitrate medium when incubated alone in the preliminary microcosm studies (section 6.3.3, Table 6.1). The culturing difficulties for the HNRB in the final study were somewhat perplexing. None of the HNRB isolates removed sulfide from the microcosms. When incubated alone, the HNRB-C did not appear to be active. The HNRB-N produced a stringy substance in the medium and did not appear to be alive. The HNRB-P attached to the serum bottle glass and remained clumped after mixing. It seems that, over time, these bacteria became increasingly sensitive to transfers into medium containing sulfide and benzoate. Most studies that include the cultivation of benzoate-utilizing SRB use modified Widdel and Pfennig medium with 2 to 5 mM benzoate and Thorstenson et al. (2002) used 20 mM acetate and 0.25 mM benzoate in their study to enrich for SRB. After monitoring benzoate tolerance for the HNRB in the enrichment process, it appeared that the HNRB were able to tolerate benzoate concentrations of approximately 1.8 mM (section 6.3.1). There was no available analytical method in this laboratory that was sensitive enough to follow changes lower than 0.1 mM benzoate so it was necessary to add approximately 1.0 mM to the cultures. It may have been beneficial to have maintained the HNRB in the presence of small amounts of benzoate in order to sustain a tolerance to benzoate. This was not done because it was thought that if the HNRB were kept in the presence of benzoate, they may develop an ability to use it as a carbon source.

The second problem was that the NR-SOB-N became difficult to maintain. This difficulty was likely because the culture became more pure and fastidious after each transfer to fresh medium. The fact that the NR-SOB-N produced N_2O in the preliminary study and did not produce N_2O in the final microcosm studies when acetylene was added to block N_2O reduction (Table 6.6) indicated that either the NR-SOB-N enrichment culture had changed over time or acetylene inhibited the culture.

The third problem was that the NR-SOB-P and -N and the HNRB were sensitive to sulfide concentrations higher than 0.8 mM when incubated alone in various media. If bacteria cannot incidentally oxidize sulfide (Paul and Clark 1996) or use it as an energy source, the sulfide can act as a poison by affecting electron transport and by binding to iron and making it unavailable for the bacteria (Reis et al. 1992, Beauchamp et al. 1984). The low concentration of sulfide in the CSB(2) medium (0.8 mM) made it difficult to detect the small concentration changes for decreased NO_3^- and increased $SO_4^=$ that occurred during sulfide oxidation.

The fourth problem was that there were difficulties during enumerations using the MPN procedures. A MPN index could not be obtained for 10 out of 108 MPN procedures. These included one procedure for HNRB (HNRB-P, day 0), one for NR-SOB-P, two for NR-SOB-N and six for SRB. The results for the HNRB and NR-SOB were not surprising. The HNRB-P attached to surfaces and was difficult to mix in the dilution blanks used for the MPN procedures. The NR-SOB-P and -N were sometimes difficult to grow in MPN enumerations. The SRB difficulty was likely due to inexperience with enumerations of SRB in pure culture. Even though the SRB medium for the MPN procedure was prepared anaerobically and reduced with sulfide and dithionite, some tubes may not have been sufficiently reduced to support the growth of low numbers of SRB that were transferred from freshly prepared dilution blanks.

The fifth problem involved the challenges associated with combining three types of bacteria (HNRB, NR-SOB and SRB) in microcosm studies. Studies with oil field isolates generally combine two types of bacteria (Telang et al. 1999, Greene et al. 2003). The study described in section 6.3.4 may be a closer representation of planktonic oil field bacterial interactions than the co-culture studies. However, combining and monitoring three types of bacteria resulted in unexpected culturing difficulties.

The sixth problem was the fact that the SRB used acetate (Widdel and Pfennig 1984). It was difficult to find suitable SRB for the final study in section 6.3.4, and due to time restraints, the *D. multivorans* strain was not fully characterized. The fact that the SRB used acetate complicated the interpretation of the results for the double and triple culture microcosms with the HNRB-C (Table 6.3). It was thought that when the SRB was cultured with an actively growing HNRB, the loss of NO_3^- would indicate that the HNRB was active. For oil field C, in the double culture microcosm, SRB plus HNRB-C, there was no clear evidence that the HNRB-C was active in the microcosm because there was no obvious loss of NO_3^- or increase of NO_2^- (Figure 6.1h). As well, in the triple culture microcosm for oil field C (Figure 6.1b), both the HNRB-C and NR-SOB-C could produce NO_2^- from nitrate reduction. In this case, the amount of NO_3^- loss indicated that both NRB were active and the MPN results showed that only HNRB-C and not SRB increased in number. These results were used to infer that the HNRB-C isolate was active in the triple culture microcosm.

The oil field NR-SOB behaved as expected from the preliminary microcosm studies (section 6.3.3, Table 6.1). In all cases, they survived the transfer to the CSB(2) medium for the final microcosm studies (section 6.3.4). They all removed sulfide in the double culture microcosms with SRB but only the NR-SOB-C removed sulfide in the triple culture microcosm before 14 d (Figure 6.1b). The NR-SOB-C was very aggressive, quickly removed sulfide (Figure 6.1c) and was easily enumerated using the MPN procedures. The NR-SOB-P was somewhat aggressive in removing sulfide when incubated alone (Figure 6.2c) but could not remove sulfide in the double culture microcosm when incubated with HNRB-P (Figure 6.2f) or in the triple culture microcosm (Figure 6.2b). It appeared to be affected by the HNRB-P isolate (Figure 6.2f). The NR-

SOB-N became slow at removing sulfide when incubated alone (Figure 6.3c) and only removed sulfide in the presence of SRB before 14 d (Figure 6.3g).

The results from this study suggest that the slow or fast removal of sulfide in oil fields is related to the particular NR-SOB from each oil field. The NR-SOB results from the enrichment cultures are similar to the results obtained from the nitrate-amendment studies described in Chapter 4 and Eckford and Fedorak (2002c). For example, in section 6.3.4.3, NR-SOB-N removed only 80% of the sulfide in the microcosm when incubated alone (Figure 6.3c) and took 5 and 28 d to remove sulfide when incubated in the double culture microcosm with SRB (Figure 6.3g) and in the triple culture microcosm (Table 6.6), respectively. The nitrate-amended oil field water from oil field N, described in Chapter 4 and Eckford and Fedorak (2002c), did not become depleted of sulfide until day 27 (Figure 4.4a). As well, in the nitrate-amendment studies, described in Chapter 4, when NO_3^- was added to microcosms containing waters from oil fields C and P (Figures 4.2a and 4.1a, respectively), the sulfide was removed by day 4. This is similar to the results shown in this study where NR-SOB-C and NR-SOB-P enrichment cultures removed sulfide from microcosms by day 1 when incubated alone and in the double culture microcosms with SRB (Figures 6.1c, 6.1g and Figures 6.2c, 6.2g, respectively). This dissertation has described sulfide removal from oil field waters in microcosm studies after NO_3^- addition. The results show that oil field waters C, N and P contain planktonic NR-SOB that can remove sulfide and HNRB that can produce intermediate nitrate-reduction products. These NRB were shown to be stimulated in the presence of NO_3^-. Their stimulation could be used to remove sulfide and inhibit SRB activity.

The results described in Chapter 4 (Eckford and Fedorak 2002c) indicated that HNRB may be involved in the quick removal of sulfide in oil field waters. This was not evident from the single culture microcosms described in this study (section 6.3.4) and shown in Figures 6.1e, 6.2e and 6.3e. It could be that the results described in Chapter 3 (Eckford and Fedorak 2002b) and Chapter 4 may have to do with the oil field waters themselves. When the waters were collected for the studies in Chapters 3 and 4, oil field P was not being treated with biocides. For this oil field water, there was quick sulfide removal and an increase in HNRB by day 38 after nitrate amendment. Oil fields N and C were receiving biocides. The biocides feed was not turned off for oil field C and there were problems with the triplicate microcosms in the nitrate-amendment studies (section 4.3.2). The biocides feed for oil field N was turned off 1 week prior to sampling in order to minimize the effects of biocides on the bacteria used in the study. In this case, there may have been residual biocides in the water sample or a change in the reservoir environment after the biocides were turned off. The change may have affected the HNRB more than the NR-SOB. As well, sulfide in the oil field water may have become toxic to HNRB because the NR-SOB from oil field N were very slow at removing sulfide This toxicity may have resulted in the decreased response of HNRB after nitrate amendment.

On the other hand, there are some results from this study and the Appendix that show how HNRB may be useful in removing sulfide in nitrate-amended environments. HNRB produce NO_2^- and N_2O from nitrate reduction and these products may be involved in sulfide control and removal. When the HNRB isolates grew well in the preliminary microcosm studies (section 6.3.3), they all produced NO_2^- (Tables 6.1) and N_2O. Except for HNRB-P, the medium in the microcosms turned pink as quickly as the medium in the microcosms with the NR-SOB. The amount of NO_2^- produced by the HNRB isolates was more than that produced by the NR-SOB enrichment cultures (Table 6.1). If there is sufficient CO_2, NO_3^-, acetate and a low concentration of sulfide in an anaerobic environment, HNRB and chemolithotrophic NR-SOB will reduce NO_3^-. The chemolithotrophic NR-SOB will only reduce NO_3^- as long as sulfide is present because sulfide is the energy source for NR-SOB. The HNRB will continue to reduce NO_3^- as long as acetate is present because acetate is used as both carbon and energy source (Kuenen 1989). In this case, the HNRB will produce the largest amount of nitrate-reduction end products.

In the double culture microcosm, when HNRB-C was combined with NR-SOB-C, there was more N_2O produced than when either culture was incubated alone (section 6.3.4.1, Table 6.2). Likewise, when HNRB-P was combined in the microcosm with NR-SOB-P, there was more N_2O produced than when the HNRB-P was incubated alone (section 6.3.4.2, Table 6.4).

Nitrous oxide could have affected the SRB in the oil field C microcosm studies. There was an increase in the SRB number and sulfide concentration for the single culture SRB microcosm and double culture microcosm, SRB plus HNRB-C, shown in Figures

6.1d and 6.1h, respectively. In these microcosms, no NO_2^- was produced and no or very little N_2O was detected when acetylene was added (Table 6.2). On the other hand, there was no increase in the SRB number for the double (SRB plus NR-SOB-C) and triple culture microcosms shown in Figures 6.1g and 6.1b, respectively, even though the SRB produced sulfide in the triple culture microcosm after NO_2^- was removed (Figure 6.1b). In these double and triple culture microcosms, there was a moderate to large amount of N_2O detected when acetylene was added (Table 6.2). Even though there was no acetylene added to the microcosms used for chemical analyses, it was assumed that N_2O may have been present in the microcosms where the SRB numbers did not change.

Other studies have mentioned that NO_2^- and N_2O may be involved in inhibiting SRB activity. In their field test at the Coleville, Saskatchewan oil field, Jenneman et al. (1999) reported that sulfide levels at injector and producer wells declined between 42 and 100% after NO_3^- was added to oil field injector wells. These authors noted that numbers of indigenous NR-SOB increased and SRB remained unchanged or decreased slightly. Their conclusions were that stimulation of indigenous beneficial bacteria has the "potential application as a cost-effective, low toxicity means to remove and control sulfides" in oil reservoirs. Nitrous oxide levels were not determined in the field and no appreciable NO_2^- was detected from field samples. These authors thought that NO_2^- could have caused the inhibitory effect to the SRB. Reinsel et al. (1996) mentioned that oxidized conditions, resulting from the formation of nitrogen oxides, may cause inhibition of sulfide production in oil reservoirs. In their study, NO_2^-, "added directly or microbially produced from NO_3^-, inhibited the production of sulfide by SRB". Myhr et al. (2002), studying the effect of NO_3^- addition on microbial H_2S production in a seawater-flooded oil field reservoir model column with crude oil as carbon and energy source, suggested that NO_2^- inhibited sulfide production by SRB. In the study, they measured NO_2^- but did not mention N_2O. As well, Greene et al. (2003) suggested that inhibition of SRB by NR-SOB is caused by NO_2^- production unless the SRB contain nitrite reductase (Nrf) activity. On the other hand, Jenneman et al. (1986) looked at the inhibition of biogenic sulfide production in dilute sewage sludge. They measured NO_2^- and N_2O and suggested that it was N_2O that raised the redox potential.

The effect of NO_2^- and, or N_2O on sulfide removal was demonstrated in the microcosm studies. For oil field C, during the microcosm studies described in section 6.3.4, in the double culture microcosm, NR-SOB-C plus HNRB-C, there was a transient increase in NO_2^-, N_2O was detected, sulfide was removed and the medium in the microcosm became pink when shaken (Figure 6.1f and Table 6.2). The pink color could have been due to dissolved N_2O in the microcosm. The pink color is caused by a change of the redox indicator (resazurin) when it becomes oxidized (Section 3.2.3). In the NR-SOB-C single culture microcosm and the double culture microcosm, NR-SOB-C plus SRB, NO_2^- accumulated, the medium turned pink, sulfide was removed and N_2O was detected (Figures 6.1c and 6.1g, respectively; Table 6.2). Although N_2O was detected in separate microcosms with acetylene, it is likely that both NO_2^- and N_2O were present in the oil field C microcosms when sulfide was removed. When the SRB were incubated with HNRB-C (Figure 6.1h), no NO_2^- was detected and sulfide production by the SRB was not inhibited. In this case, only a scant amount of N_2O was detected in the microcosm with acetylene and the medium remained colorless (Table 6.2).

For oil field P, during the microcosm studies described in section 6.3.4, the medium in the HNRB-P single culture microcosm became slightly pink when shaken and there was no decrease in sulfide (Figure 6.2e, Table 6.4). The appearance of NO_2^- was transient so the pink color may have been due to the presence of dissolved N_2O. A pink color also developed in the medium after the HNRB-P plus NR-SOB-P double culture microcosm was shaken. Sulfide was not removed in this microcosm (Figure 6.2f, Table 6.4). Again, there was a transient increase in NO_2^-. The MPN value for NR-SOB-P decreased significantly and this decrease, if accurate, may explain why the sulfide was not removed (Figure 6.2f). On the other hand, the medium in the single culture microcosm with NR-SOB-P and the double culture microcosm, NR-SOB-P plus SRB became very pink on day 1 (Table 6.4). There was a complete loss of sulfide, continuous presence of NO_2^- and no N_2O was detected in the microcosm with acetylene (Figures 6.2c, 6.2g). The results indicate that in the oil field P microcosms, when sulfide was removed, only NO_2^- was present.

For oil field N, during the microcosm studies described in section 6.3.4, the NR-SOB-N slowly decreased the sulfide concentration in the single culture microcosm

(Figure 6.3c). The medium in the microcosm turned yellow (Table 6.6). In the double culture microcosm SRB plus NR-SOB-N (Figure 6.3g), sulfide was completely removed by day 5 and the medium turned pink (Table 6.6). In these microcosms, NO_2^- accumulated and no N_2O was detected in the microcosms with acetylene. The results from the oil field N microcosm studies indicate that only NO_2^- was present in the microcosm with SRB and NR-SOB-N when sulfide was removed before day 14. As shown in the Appendix, the combination of NO_2^- and sulfide may have produced the yellow color in the medium for the NR-SOB-N microcosm. It is not known what caused the medium to turn yellow in the other microcosms with no detectable NO_2^- (Table 6.6).

The transient effect of NO_3^- reduction on sulfide was demonstrated in the triple culture microcosm for oil field C (section 6.3.4.1). Here sulfide was quickly removed, NO_3^- was reduced and the medium turned pink. Nitrate became limiting and NO_2^- did not accumulate. The medium in the microcosm lost the pink color and the SRB actively produced sulfide (Figure 6.1b). The results from this microcosm indicate that the presence of either or both NO_2^- and N_2O initially increased the redox potential producing the pink color in the medium on day 1 (Table 6.2). The transient effect of NO_3^- on sulfide removal was mentioned by Reinsel et al. (1996). They state that organic matter remaining after NO_3^- depletion could be used by SRB to reduce $SO_4^=$ to sulfide.

The limitations put on the study described in section 6.3.4 may have selected against results that would show how HNRB are involved in decreasing sulfide concentrations when NO_3^- is present. Removing some of the limitations may provide better results but may not provide clear answers as to bacterial roles in sulfide removal. Petroleum reservoirs contain many different types of bacteria under anaerobic conditions with oxygen, NO_3^- and ferric iron generally absent (Magot et al. 2000). The use of mixed anaerobic HNRB cultures grown in medium with no NO_3^-, prior to nitrate-amendment studies, may be more like the nitrate-amended oil reservoir conditions. As well, a similar study using a mixture of HNRB may be needed to show if HNRB have a role in sulfide removal. The results shown in this thesis are based on planktonic oil field bacteria which is only a very small bacterial population of an oil reservoir (McInerney and Sublette 2002). Oil field bacteria are often present in biofilms which could give protection for HNRB (Costerton 1995).

Some interesting observations have come from this work. The fact that the isolation of the bacteria was difficult is not a surprise. The methods used for enrichment of NRB in this study may have been too stringent to culture some sulfide-tolerant or sulfide-oxidizing NRB that were present in the oil field waters. Amann et al. (1995) mentioned some problems with culturing and enumerating environmental bacteria. They emphasize that most bacteria can be visualized microscopically but do not form visible colonies on plates. They go on to say that two different types of cells contribute to the silent but active majority. First, are the known species that are in a nonculturable state or are not suited for the methods applied. Second, are the unknown species that have not been cultured due to the lack of suitable methods. Molecular methods are often used for studies involving non-culturable bacteria, but the fact that NRB processes are performed by a great diversity of bacterial strains from all the major physiological groups does not allow for the use of the classic approach of targeting the ribosomal gene. However, Michotey et al. (2000) were able to detect cytochrome *cd*$_1$-denitrifying bacteria in environment marine samples using polymerase chain reaction (PCR) primer sets that they had developed.

For oil fields P and N, there was no increase in sulfide or changes in other anions when the SRB were incubated in the double culture microcosm with HNRB (Figures 6.2h and 6.3h, respectively). For oil field P, the HNRB was active in the single culture microcosm (Figure 6.2e) and for oil field N, the HNRB was inactive in the single culture microcosm (Figure 6.3e). These results were not like the results for the oil field C double culture microcosm, SRB plus HNRB-C, where SRB activity was noticeable. In this microcosm there was a decrease of $SO_4^=$ and increase in sulfide concentrations (Figure 6.1h). In all cases, when SRB were incubated alone, they actively produced sulfide (Figures 6.1d, 6.2d, 6.3d). There is no obvious explanation for the oil field P and N double culture microcosm results other than the fact that the medium in the microcosms contained 10 mM NO_3^- (oil field C microcosm medium had 3 mM NO_3^-). There is also no obvious explanation for the inactivity from days 0 to 7 in the triple culture microcosm for oil field P (Figure 6.2b). The presence of a possible inhibitory substance in the microcosms or NO_2^- and N_2O that may have come from the unwashed HNRB and NR-SOB inoculums could be used as an explanation. However, for oil fields P, N and C, the same medium was used to grow the cultures before inoculation into the microcosms, and the same methods were used to inoculate the cultures for the microcosm studies.

The fact that HNRB did not appear to be actively involved in removing sulfide in the microcosm studies, described in this chapter, does not give conclusive evidence that active populations of obligate and facultative types of HNRB in oil reservoirs do not have a role in the removal of sulfide. In the present study (sections 6.3.3 and 6.3.4), it was shown that the HNRB isolates produced NO_2^- and N_2O, and in the Appendix (Figure A.2), it is shown that NO_2^- can abiotically remove sulfide from culture medium. It is also mentioned in the Appendix that N_2O can abiotically remove sulfide from culture medium.

As mentioned before, one mechanism which both types of NRB could use to remove sulfide and inhibit SRB in oil fields could be the production of the intermediate products. In the microcosm studies from sections 6.3.3 and 6.3.4, the NR-SOB enrichment cultures all produced NO_2^- (Tables 6.1, 6.2, 6.4, 6.6) and only NR-SOB-C consistently produced N_2O (Table 6.2). When the NR-SOB enrichment cultures were combined with the SRB in the double culture microcosms, sulfide was removed and the SRB were inhibited (Figures 6.1g, 6.2g, 6.3g). The CSB(2) medium in the microcosms turned pink (Tables 6.2, 6.4, 6.6) which was likely due to the presence of NO_2^- for microcosms with NR-SOB-P and NR-SOB-N and a possible combination of NO_2^- and N_2O for the microcosm with NR-SOB-C.

In the microcosm studies from section 6.3.3, the HNRB all produced NO_2^- and N_2O. In the microcosm studies from section 6.3.4, the microcosm with HNRB-C plus NR-SOB-C had a transient increase in NO_2^-, sulfide was removed (Figure 6.1f) and there appeared to be dissolved N_2O in the medium (Table 6.2). In this case, the NR-SOB-C likely removed the sulfide and both HNRB-C and NR-SOB-C produced the NO_2^- and N_2O.

For the double culture microcosm, HNRB-P and NR-SOB-P (Figure 6.2f), the NR-SOB-P and HNRB-P did not remove the sulfide, and it appeared as though there was dissolved N_2O in the medium (Table 6.4). This result was perplexing because if N_2O can remove sulfide, as shown in the Appendix, why was the sulfide concentration not reduced in this microcosm? In this case, it could be that the concentration of N_2O in the microcosm was not high enough to remove the sulfide in 14 d. A portion of the N_2O may have been reduced to N_2 by the HNRB-P because the NO_3^- and NO_2^- concentrations were below detection by day 5 (Figure 6.2f), acetate was present (Table 6.5) and there was no acetylene in the microcosm to block N_2O reduction. It could also be that N_2O plus NO_2^- were needed for the abiotic removal of sulfide or that the microcosm was affected by sonication so that any results after day 7 were invalid.

Suppose an oil field, containing HNRB and NR-SOB, was nitrate amended and the HNRB population dramatically increased and produced large amounts of NO_2^- and N_2O. From the results shown in this study and the Appendix, the NO_2^- and N_2O could possibly remove sulfide as well as raise the redox potential of that environment and inhibit sulfide production by SRB. In Chapter 4 (Eckford and Fedorak 2002c), a hypothesis was made from the nitrate-amendment results for oil fields P, C and N. The hypothesis stated that "in order to hasten sulfide removal, an active HNRB population is required to either out-compete heterotrophic SRB for carbon source and, or produce end products, from nitrate reduction, that raise the redox potential and inhibit SRB". For oil field N, during the nitrate amendment studies, the NR-SOB-N number increased, the HNRB-N number did not increase (Figures 4.5a, 4.5b) and sulfide was slowly removed (Figure 4.4a). If the HNRB population from oil field N had dramatically increased and produced large amounts of NO_2^- and N_2O after nitrate amendment, described in Chapter 4, would the sulfide have been quickly removed?

Chapter 3 (Eckford and Fedorak 2002b) documented the presence of HNRB and NR-SOB in the oil field waters that were nitrate amended and Chapter 4 (Eckford and Fedorak 2002c) showed that sulfide was removed in these waters after NO_3^- addition. From these results, it can be stated that in a sulfide-containing oil field with NR-SOB and HNRB, sulfide would be removed and sulfide production would be inhibited after the addition of NO_3^-. It is thought that NR-SOB would reduce NO_3^-, produce NO_2^- and, or N_2O, while removing sulfide. After sulfide depletion, the NR-SOB would be inhibited if they can not use an alternate energy source. As well, the HNRB would reduce NO_3^-, and continually produce NO_2^- and, or N_2O as long as NO_3^- and their carbon source do not become limiting. Again, from the results in the Appendix, it can be stated that the continual production of the NO_2^- and, or N_2O could remove sulfide and prevent SRB from producing more sulfide (Jenneman et al. 1986). Of course, unlike laboratory microcosms, oil field environments are large complex systems, and in an oil field there would be many variations on the sulfide removal theme. It is likely that the SRB would not be completely inhibited after nitrate amendment as shown in field studies (Table 1.6). Some sulfide may remain and the NR-SOB would oxidize the sulfide and produce nitrate-reduction intermediate products. What the results from section 6.3.3 have shown is that HNRB acting alone or together with NR-SOB would produce much more NO_2^- and, or N_2O than NR-SOB alone and could possibly contribute to sulfide removal and SRB inhibition.

Two different examples of NO_3^- addition to oil fields show how each oil field responds differently to NO_3^- addition. At the Skjold oil field in the North Sea, Larson (2002) found that during a fieldwide trial over 3 months with an average 2.8 mM NO_3^- daily injection rate, there was a significant decrease in sulfide at one production well. The mechanism for sulfide removal by NO_3^- was thought to be "nitrite build-up or biological oxidation of sulfide due to the activity of NR-SOB". There was evidence that the SRB population was only inhibited and became active again when NO_3^- was removed. Jenneman et al. (1999), on the other hand, added NH_4NO_3 and NaH_2PO_4 continuously to two injector wells at Coleville, Saskatchewan for 50 d. They stated that following chemical injection NRB numbers increased at both injectors while SRB remained at pre-treatment levels. When chemical injection was halted, the NRB population declined rapidly to near pre-treatment levels and the SRB population decreased to below pre-treatment levels. The authors could not explain the SRB result but thought that it was the "result of a shift in the local sessile populations from one dominated by SRB to one dominated by NR-SOB".

For the oxidation of sulfide, higher concentrations of NO_2^- and N_2O appear to be required if the process is strictly abiotic than if NO_2^- and N_2O are produced from NRB during the reduction of NO_3^-. Abiotic removal of sulfide using NO_2^- and N_2O is described in the Appendix. In a study described by Reinsel et al. (1996) using crushed Berea sandstone columns with oil field produced water consortia incubated at 60°C, the authors reported that sulfide production was inhibited when the column was treated with 3.6 mM NO_3^- for over 2 months. When NO_3^- was removed, SRB activity resumed. Once the column was treated with NO_3^-, as little as 0.71 mM NO_3^- was required to inhibit sulfide production and the inhibition was maintained with as little as 0.36 mM NO_3^-. These authors continue to say that the stoichiometric calculations show that about 1.6 mM NO_3^- is required to oxidize each mmol of sulfide so that "with a steady state sulfide production of 3.1 to 3.9 mM, this would require continuous injection to 5.3 to 6.4 mM nitrate". The calculated amount of NO_3^- required to oxidize sulfide was much higher than the concentration of nitrate needed in their study to inhibit SRB activity. For the Reinsel et al. (1996) study, the types of bacteria in the oil field produced water consortia were not determined. If some of the sulfide in oil reservoirs is removed abiotically by NO_2^- and N_2O after nitrate amendment, the HNRB and NR-SOB populations together could produce sufficient concentrations of these nitrate-amendment products for its removal.

There appears to be no reports in literature of any molecular mechanism by which oil field heterotrophic or mixotrophic bacteria oxidize sulfide while using NO_3^- as an electron acceptor. Reports from other environments regarding the oxidization of inorganic sulfur compounds have been made for heterotrophic bacteria along with mixotrophic, sulfur-oxidizing, chemolithotrophic bacteria that use organic carbon for their carbon source. It is thought that mixotrophs, growing exclusively on organic compounds, benefit from sulfide oxidation during detoxification of H_2O_2 which would presumably be formed under aerobic conditions. In soils, heterotrophic bacteria are thought to be the primary oxidizers of inorganic sulfur in neutral and alkaline soils because of their diversity and large populations. In this case, heterotrophic bacteria do not gain energy from the sulfur oxidation and the transformations are thought to be incidental to the major metabolic pathways. Intermediate products of sulfide oxidation (thiosulfate and tetrathionate) accumulate and sometimes sulfate is formed (Paul and Clark 1996). It is not known which enzyme catalyzes sulfide oxidation, in vivo, for most organisms. It is thought that sulfide is oxidized to sulfur by two types of sulfide dehydrogenase; one is membrane associated and the other is periplasmic. Most of the sulfide oxidation work has been done on phototrophic and chemolithotrophic bacteria (Shibata and Kobayashi 2001, Griesbeck et al. 2000).

Kelly et al. (1997) describe two fundamental oxidation processes that exist for energy production from the oxidation of sulfur, sulfide and thiosulfate: (i) the

S_4intermediate (S4I) pathway, involving polythionates and occurring in the periplasm and cytoplasm of a cell and (ii) the *Paracoccus* oxidation pathway (PSO), not involving polythionates and occurring in the periplasm of a cell. In the PSO pathway, thiosulfate is oxidized via an enzyme system with five components and no sulfite or other thiosulfate intermediates are formed. The PSO pathway may be used by facultative heterotrophs, such as *Paracoccus* (Kelly et al. 1997) and some *Paracoccus* like *P. pantotrophus* are capable of denitrification (Robertson and Kuenen 1983, Rainey et al. 1999). The S4I and PSO pathways describe the oxidation of thiosulfate and do not mention sulfide. White (1995) gives a pathway for inorganic sulfur oxidation with sulfide. In this case, sulfide reacts with reduced glutathione to from a linear polysulfide. Sulfur atoms are removed from the polysulfide one at a time during the oxidation to sulfite and then sulfate.

The limitations imposed on the HNRB and NR-SOB during enrichment and cultivation for the study in this chapter selected for only one type of HNRB and may have hindered the growth of various types of NR-SOB from the oil field waters. Most of the media that were used for this research project were designed to grow specific types of NRB. This was important in order to distinguish among the various types of NRB that could be present in oil field waters. In all cases, NO_3^- was included in the media to select for NRB. Defined media for chemolithotrophic NRB were prepared anaerobically and contained no carbon source. When planktonic oil field HNRB were enumerated, they appeared to grow well in the aerobically prepared nutrient broth-nitrate medium. The growth in this medium may have also included other types of bacteria that used the rich organic substrate. Although it could be argued that the nutrient broth-nitrate medium could select for certain bacteria because of competition for substrates during the 30-d incubation period, there is also a potential for synergistic or mutualistic growth which may occur in oil field environments. The success of this medium for growing heterotrophic bacteria from oil field waters, suggested that oil fields are harsh environments and that oil field HNRB may require other bacteria in order to grow well. The problem with using nutrient broth-nitrate liquid medium for HNRB studies is that there is no way of identifying specific bacterial functions from a mixed environmental sample and it is often difficult to measure substrate changes in the organic medium. The results from the studies described in this chapter showed that HNRB become fastidious and unpredictable in pure culture studies using defined conventional mineral medium, so that the studies became more centered on bacterial growth than on bacterial function.

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7. Overall discussion and suggestions for further research

7.1 Overall discussion

The mainspring for studying nitrate amendment to oil field waters is the need to investigate a suitably safe and economical method to eliminate microbially-produced sulfide in oil field settings. Sulfide causes environmental and economic difficulties for the petroleum industry and the methods used to control sulfide in oil field settings can often be problematic (American Petroleum Institute 1995, Cord-Ruwisch et al. 1987). The complications associated with sulfide and the use of NO_3^- to control sulfide in an environment are not new.

Chapter 1 described investigations where NO_3^- addition to wastewater successfully controlled odor and sulfide production (Lawrance 1950, Poduska and Anderson 1981, Jenneman et al. 1986, Londry and Suflita 1999, Sublette et al. 1994). Chapter 1 also described laboratory and field studies, reported prior to the initiation of the investigations documented by this thesis, in which NO_3^- was evaluated as a control for sulfide in oil field settings. The laboratory studies were done, under various test conditions using columns and cores (McInerney et al. 1992, McInerney et al. 1996, Reinsel et al. 1996, Jenneman et al. 1996), enriched nitrate-reducing bacteria (NRB) (McInerney et al. 1992, McInerney et al. 1996) and natural microbial communities from produced waters (Gevertz et al. 1995, Jenneman et al. 1996, Wright et al. 1997).

In field studies from Oklahoma, USA (McInerney et al. 1993) and Saskatchewan, Canada (Gevertz et al. 1995; Jenneman et al. 1997, 1999), NO_3^- was injected into the oil fields. In the Oklahoma field, there were diverse populations of bacteria, including NRB. After nitrate injection, the researchers reported that the sulfide levels decreased. On two occasions, NO_3^- was injected into the Saskatchewan field, and on both occasions, the researchers mentioned that sulfide decreased and there were increases in the NRB population.

Several studies, discussed in Chapter 1, have investigated NRB in oil field waters, and four studies, prior to the work documented here, enumerated NRB in oil field waters using most probable number (MPN) methods with different media formulations (Adkins et al. 1992; Gevertz et al. 1995; Telang et al. 1997, 1999). Most of the studies focussed on two chemolithotrophic nitrate-reducing, sulfide-oxidizing bacteria (NR-SOB), *Thiomicrospira* strain CVO and *Arcobacter* strain FWKO B, isolated from Saskatchewan oil field water. There had been no study that had examined different types of NRB in oil field waters or the effect that NO_3^- addition to oil field waters would have on these bacteria. Wright et al. (1997) suggested, after studying produced brines from four different carbonate formations in west Texas with reservoir temperatures of 40° to 60°C, that sulfide bioscavenging by indigenous bacteria was mediated by heterotrophic bacteria.

It is thought that NRB, in the presence of NO_3^-, can control sulfide in several ways: by out-competing heterotrophic sulfate-reducing bacteria (SRB) for electron donors, as shown with equations 1.5 and 1.6; by producing intermediate products like N_2O that raise the redox potential and inhibit SRB (Jenneman et al. 1986); by removing sulfide from aqueous environments (Jenneman et al. 1996) and by producing NO_2^- that can accumulate and inhibit some SRB (Myhr et al. 2002). The microbial mechanisms by which NO_3^- addition is used to control sulfide concentrations are discussed in Section 1.6.1.

The first goal of this study was to develop methods to enumerate planktonic heterotrophic NRB (HNRB) and chemolithotrophic NRB using MPN procedures. There are two important reasons why this was necessary. First, it was important to find methods to determine if different types of NRB are present in oil fields that are being considered for nitrate amendment. Second, during nitrate amendment to oil fields, it is prudent to be able to predict the fate of NO_3^- and which sulfide-controlling mechanism may be used by NRB to control sulfide.

In order to accomplish this goal, media had to be used that would distinguish between the HNRB and chemolithotrophic NRB. Tiedje (1982) had described a method for cultivating HNRB using a nutrient broth-nitrate liquid medium and the diphenylamine spot test. During preliminary studies, it was found that the diphenylamine spot test, used to determine NO_3^- loss (Morgan 1930), did not always give reliable results for oil field waters. The organic nutrient broth-nitrate medium is not specific for N_2O-producing NRB, and HNRB can form other intermediate products during nitrate reduction.

Therefore, the only way to enumerate some HNRB was to monitor NO_3^- loss in MPN tubes with nutrient broth-nitrate liquid medium. Conventional methods for NO_3^- measurement were not suitable for measuring NO_3^- loss because of interference from high Cl^- concentrations and organics in the medium. The only suitable method for determining NO_3^- loss in the MPN tubes that was found to be simple, quick and reliable was a second derivative UV absorbance method. The method is an adaptation of the second derivative UV absorbance method used by Crumpton et al. (1992) for analyzing NO_3^- in fresh-waters.

The development of the second derivative UV absorbance method is detailed in Chapter 2 and Eckford and Fedorak (2002a). Basically, in order to define a given component in a complex medium, derivative spectra are used. Here, the first derivative is used to diminish broad peaks and remove constants. The second derivative defines the rates of change in the first derivative absorbance spectrum with respect to wavelength. A sharp peak in the spectrum created by NO_3^- will be emphasised at this point (Cahill 1979). Second derivative UV absorbance screening methods were developed for the determination of NO_3^- loss in MPN culture tubes for HNRB and thiosulfate-oxidizing NRB.

The MPN procedures used for NRB were developed so that enumerations were based on measurements of substrate loss and, or end product formation after a 30-d incubation period. This was done in order to determine the types of NRB in oil field waters. A MPN procedure was done with aerobically prepared nutrient broth-nitrate medium to enumerate N_2O-producing HNRB. Nitrate loss was measured using the second derivative UV absorbance method and N_2O using a gas chromatography method. Even though the nutrient broth-nitrate medium could grow many types of heterotrophic bacteria, the loss of NO_3^- and production of N_2O in the MPN tubes showed that N_2O-producing HNRB were present.

The MPN procedure for enumerating chemolithotrophic, thiosulfate-oxidizing NRB used anaerobically prepared mineral medium containing CO_2 for a carbon source and thiosulfate for an energy source. The loss of NO_3^-, measured by the second derivative UV absorbance method and presence of NO_2^- indicated that thiosulfate-oxidizing NRB were present. The MPN procedure for enumerating sulfide-utilizing NRB, or NR-SOB, used anaerobically prepared mineral medium that contained CO_2 for a carbon source and sulfide for an energy source. In this case, the tubes were incubated in an O_2-free atmosphere to prevent O_2-oxidation of sulfide. The MPN tubes were analyzed for sulfide loss and NO_2^- production. The tubes were also observed for changes in color that would indicate the presence of NR-SOB.

When the development of methods was completed, enumerations of planktonic bacteria in oil field waters were done, as described in Chapter 3 and Eckford and Fedorak (2002b). Enumerations of aerobic bacteria were done using plate counts and NRB and SRB using MPN procedures. Five oil fields in Alberta and Saskatchewan were sampled. Four of the oil fields had sulfide, in concentrations from 0.2 to 5 mM, that was detected in all or some of the produced waters. Where possible, produced water was collected from four locations at each oil field. The locations included the wellhead; source water used for waterflooding; two aboveground locations including the treater, where the produced oil field components are separated, and the storage facility, where water is collected before being returned to the oil field. These locations were chosen in order to determine sulfide concentrations throughout the oil field while following the path that the waterflood takes throughout the oil field. The oil fields are similar in that they are shallow with depths from about 800 to 1500 m, of moderate temperature, waterflooded with surface or aquifer source water and have nitrate-free produced waters. The oil fields are different in production size with production wells varying in numbers from 40 to 245 and injection wells from two to 110. The collected, produced waters had Cl^- concentrations that ranged from 0.1 to 2400 mM and $SO_4^{=}$ concentrations that were variable. In some oil fields there was evidence that $SO_4^{=}$, used by SRB to produce sulfide, may have been introduced with the source waters and in other oil fields the $SO_4^{=}$ was from the reservoir.

Briefly, oil fields B, N and P had four water samples analyzed and oil fields A and C had one aboveground water sample analyzed. NR-SOB were not enumerated for oil field A. SRB were found in wellhead or satellite water samples for oil fields B and P and in aboveground water samples for oil fields A, B, C, N and P. HNRB were found in wellhead or satellite water samples for oil fields B and P and in aboveground water samples for oil fields A, B, C, N and P. NR-SOB were not found in wellhead or satellite locations for any oil field water samples and in aboveground water samples for C, N and P. The source water for B and N contained SRB and HNRB but not NR-SOB. Oil field P source water was produced water from another field.

The second goal of this study was to monitor chemical and bacterial changes in laboratory microcosms containing nitrate-amended oil field waters. The results from the second goal would show, using laboratory-scale experiments, whether or not NO_3^- could be added to produced waters to control sulfide-producing activity in the oil fields. Chapter 4 and Eckford and Fedorak (2002c) describe the methods used to accomplish this goal. One water from each of three oil fields was chosen for nitrate amendment. The oil field water samples were taken from the locations within the oil fields that had the highest sulfide concentrations, the treater and storage locations.

Oil field waters were put into anaerobic 158-mL serum bottle microcosms. There were three sets of serum bottle microcosms containing nitrate-amended waters, non-amended waters and twice autoclaved waters for sterile controls. The microcosms were monitored and compared for chemical and bacterial changes using methods that had been developed for the enumeration of planktonic bacteria. The results from these studies showed that, in the nitrate-amended microcosms, sulfide was removed from two waters within 4 d and from one water by 27 d. The NO_3^- addition stimulated large increases in the number of chemolithotrophic NR-SOB in all oil field waters and large increases of HNRB in only two oil field waters. During this testing period, a role for HNRB in sulfide removal was not established. From these results, it was hypothesized that stimulation of HNRB is required for rapid removal of sulfide from oil field produced waters.

A third goal of this study was to evaluate a MPN enumeration method for dissimilatory ammonium-producing, nitrate-reducing bacteria (DAP-NRB). The DAP-NRB MPN enumeration method was used to complete the study of HNRB that may be stimulated by NO_3^- addition to oil field waters. The significance of the DAP-NRB study relates to the importance of predicting which oil field NRB may be stimulated by NO_3^- addition and the fate of NO_3^- after addition to an oil field. The MPN procedure included enumeration of DAP-NRB in environmental waters using dissimilatory ammonium production (DAP) medium and subsequent detection of NH_4^+ after a 30-d incubation. The description of this study is found in Chapter 5. The MPN procedure was used to detect

DAP-NRB at the same time that planktonic bacteria were enumerated in the 18 oil field waters. Using the DAP-NRB method, a total of 29 environmental water samples were enumerated and 7 of the samples had MPN results based on turbidity in the DAP medium that were statistically greater than MPN results based on detected NH_4^+. When the DAP-NRB MPN method was used during NO_3^- addition to oil field waters, 11 samples were enumerated and 8 of the samples had MPN results based on turbidity in the DAP medium that were statistically greater than MPN results based on detected NH_4^+. These results led to the stipulation that the DAP-NRB enumeration method must be interpreted with caution. Because DAP medium is not specific for DAP-NRB in a sample, there is no simple way of determining if all MPN results based on ammonium production are true, or if NH_4^+ in the higher dilution MPN tubes is removed from the DAP medium by other bacteria that could grow in DAP medium.

Four results came from the DAP-NRB study: first, twelve of eighteen oil field water samples contained DAP-NRB; second, DAP-NRB numbers were low in oil field waters; third, the DAP-NRB were seldom detected in wellhead samples and fourth, after nitrate amendment to oil field waters, it appeared as though the DAP-NRB remained a minor portion of the NRB community.

The study in Chapter 6 describes methods for the enrichment of NRB from three oil field waters. The NRB were used in laboratory microcosm studies to determine the roles of HNRB and chemolithotrophic NR-SOB during sulfide removal. The impetus for this study came from the results, shown in Chapter 4, after NO_3^- was added to water collected from oil fields. The Chapter 4 results indicated that HNRB may be involved in the quick removal of sulfide in oil field waters and there was a suggestion that "in order to hasten sulfide removal, an active HNRB population is required to either out-compete heterotrophic SRB for carbon source and, or produce end products, from nitrate reduction, that raise the redox potential and inhibit SRB". The study in Chapter 6 was designed with the idea that the heterotrophic bacteria would not compete for a common carbon source.

Enrichment cultures for HNRB were made from three sulfide-containing oil field waters using acetate in liquid nutrient broth-nitrate medium. The enrichment cultures were plated to aerobic nutrient broth-nitrate agar plates plus acetate and isolated colonies were obtained. The HNRB isolates were required to use acetate as a carbon source, produce N_2O from nitrate reduction and tolerate low concentrations of sulfide. One HNRB isolate was obtained from each oil field. In addition, three chemolithotrophic NR-SOB mixed cultures were enriched from the same oil field water as each HNRB. The NR-SOB were enriched in anaerobic mineral medium with CO_2, NO_3^- and sulfide. The chemolithotrophic NR-SOB were required to use sulfide while reducing NO_3^-. Pure NR-SOB isolates were not obtained.

A defined mineral medium was used for laboratory microcosm studies. Each HNRB and NR-SOB as well as a SRB, *Desulfococcus multivorans*, were monitored in single, double and triple culture microcosm studies. The heterotrophic *D. multivorans* was chosen to use benzoate as a carbon source and $SO_4^=$ as an electron acceptor. The microcosms were analyzed for growth, acetate and benzoate loss, NO_3^- loss, production of nitrate-reducing end products and sulfide loss.

The results from the study in Chapter 6 indicated that each oil field had unique, culturable NR-SOB that could remove sulfide. There was no clear evidence for a specific role that HNRB may have for the removal of sulfide. The HNRB consistently produced NO_2^- and N_2O. Both of these nitrate-reduction products were shown to remove sulfide in abiotic studies (shown in the Appendix). Sulfide was removed in all double culture microcosms with NR-SOB and SRB and the removal was consistent with the results given for each nitrate-amended oil field water in Chapter 4 (Eckford and Fedorak 2002c).

The overall conclusion from this research project is that waterflooded oil fields can contain sulfide, SRB and NRB. Specifically, oil fields C, N and P contained SRB and sulfide. These oil fields also contained NR-SOB that were stimulated after NO_3^- addition. The oil fields also contained HNRB and these increased in number for oil fields C and P after NO_3^- addition. The HNRB and NR-SOB produced intermediate products from nitrate reduction that can be used to inhibit SRB activity. The results given for this work show that NO_3^- can be used to remove sulfide from oil fields C, N and P. On the other hand, oil fields A and B were not considered sour even though they both contained SRB. Oil field A had 0.3 mM sulfide at an aboveground location and oil field B had no detectable sulfide.

Preliminary laboratory work must be done before a nitrate amendment program is initiated in an oil field to control sulfide production. Laboratory work should include the enumeratation of various types of NRB so that mechanisms used by NRB to inhibit SRB activity can be predicted. As well, the concentration of NO_3^- used for a particular oil field should be determined. Three oil field studies reported different NO_3^- concentrations that were used to inhibit SRB activity. These NO_3^- concentrations were: 6.5 mM over 50 d for the Coleville, Saskatchewan oil field (Jenneman 1999); 0.25 mM over 32 months for the Veslefrikk, North Sea oil field (Thorstenson et al. 2002) and 2.8 mM over 3 months for the Skjold, North Sea oil field (Larsen 2002).

It is also important to consider what conditions should be used for oil fields when NO_3^- is added. Larson (2002) presented results from laboratory work that was done prior to a field application of NO_3^-. During preliminary coreflood and laboratory loop studies, he reported conditions that were considered before a field nitrate amendment trial was done at the Skjold oil field in the North Sea. The conditions included testing NO_3^- under various oil field settings. Nitrate was examined for its ability to remove sulfide solids from water injection well equipment and biolfilm slime from metal surfaces. It was also determined if the use of NO_3^- would reduce the oil field chalk matrix rock permeability and whether the combination of sulfide and NO_3^- or sulfide and NO_2^- would result in corrosion-causing products. Compatibility of NO_3^- with the oil field injection and production chemicals was tested. As well, investigations were done to determine whether adding NO_3^- to the mixed microbial consortia would increase sulfide production at the start or end of the NO_3^- addition process.

Two of the three oil fields that were studied in this dissertation contained a sufficient supply of organic substrates so that the addition of NO_3^- to the sour oil field waters resulted in the stimulation of HNRB in the two oil field waters. Other studies have demonstrated that all oil fields do not contain the organic substrates needed by NRB to inhibit SRB activity. Wright et al. (1997) showed in their study of four west Texas brines from carbonate reservoirs with temperatures from 40 to 60°C that the addition of NO_3^-, PO_4^{3-}, acetate, formate, vitamins, amino acids and trace metals "may be needed to obtain optimal oxidation rates by indigenous heterotrophic sulfide-oxidizing bacteria" especially in oil fields where extensive waterflooding had diluted the water soluble organics.

Thorstenson et al. (2002) mentioned that the Veslefrikk oil field in the North Sea may be carbon limited. These studies show that it is necessary to analyze oil field waters for the nutrients needed by indigenous NRB after NO_3^- addition to an oil field. It is important for water analyses to be done before the initiation of nitrate amendment to an oil field, because the addition of nutrients other than NO_3^- would increase the cost of nitrate amendment for the oil field.

7.2 Suggestions for future research

7.2.1 Characterizing organic and inorganic components in oil field waters

In Chapter 4, it was suggested that "determining the types and quantities of the organic compounds dissolved in the produced waters would have provided valuable information to help assess which electron donors were key to the anaerobic processes that took place after nitrate amendment". Characterizing organic compounds and trace elements in oil reservoirs and adding these components to defined medium would help to make the interpretation of results from studies, using defined medium, more specific than speculative. The added components may allow for the cultivation of more bacterial types that could be used in studies using oil field isolates and enrichment cultures. The results from these studies may provide a more accurate description of sulfide removal in oil reservoirs. Magot et al. (2000) stated that oil reservoirs can contain many different types of electron donors and acceptors. Potential electron donors can include H_2, and many organic molecules specific to an oil reservoir. Resins and asphaltenes may contain heteroatoms of nitrogen, sulfur and oxygen and these may provide essential elements for growth by oil reservoir bacteria. The culturing problems encountered during enrichment of oil field NR-SOB in Chapter 6 and the fact that different types of sulfur-oxidizing bacteria like thiosulfate-oxidizing NRB were not detected in the studies described in Chapters 3 and 4 (Eckford and Fedorak 2002b, 2002c) may have been due to the inadequacy of the culture media. Barth (1991) described analytical methods for organic acids and inorganic ions in oil reservoir waters using "isotachophoresis, an electrophoretic technique that separates ionic compounds by their acid strength".

7.2.2 Using molecular biology methods to determine oil field microbial diversity

The use of molecular biology methods for oil field waters would be of benefit in determining the diversity of oil field microbial populations. The methods for determining microbial diversity should not rely entirely on culturing techniques because many of the bacteria could be either unculturable or difficult to culture. Some genetic methods to be used may include denaturing gel gradient electrophoresis or terminal restriction fragment length polymorphisms that identify community patterns of 16S rDNA. Madsen (2000) suggests that these methods "have been applied routinely in microbial ecology and are suitable for gathering information about subsurface microorganisms". As well, new improved techniques for analyzing functional diversity in an environment are emerging which use DNA microarray methods for the detection and quantification of functional genes in the environment (Taroncher-Olderburg et al. 2003). Using a combination of molecular and cultural methods may be needed to define the microbial communities in oil reservoirs.

7.2.3 Characterizing bacteria that produce sulfide in oil reservoirs

Sulfide-producing activity in oil field settings has been largely attributed to sulfate-reducing, lactate-oxidizing SRB. The fact that this research project demonstrated that there were different types of NR-SOB in three oil field waters should also be applied to SRB. Devereux and Stahl (1993) list several species of SRB that do not use lactate, and McInerney and Sublette (2002) mention that *Shewanella* species, capable of reducing sulfite and thiosulfate instead of $SO_4^{=}$, may be the most numerous bacteria that reduces sulfur oxyanions to sulfide in some oil fields.

The studies described in this thesis were designed to use the same medium for enumerating and monitoring each type of planktonic bacteria, before and after NO_3^- addition to oil field waters. If the same medium is used for each bacterial type, changes could be followed over time. Oil field nitrate-amendment studies should be done to include media that would show that more than one electron donor and acceptor could be used by sulfide-producing bacteria.

7.2.4 *Analyzing sulfide oxidation products produced after NO_3^- addition to oil field waters*

In Chapter 4, observations were made of the color changes in modified CSB medium for the enumeration of NR-SOB from oil field N. In this case, the modified CSB medium became yellow and then pink. Subsequent work on oil field N (documented in Chapter 6) showed that the NR-SOB-N produced a yellow color in mineral medium with sulfide. In the NR-SOB-N microcosm (Chapter 6), sulfide was partially removed and 0.5 mM of NO_2^- was detected. The NR-SOB-N did not produce N_2O. For the other two oil field NR-SOB, described in Chapter 6, the medium in the microcosms turned pink and sulfide was removed. In one case, 0.9 mM NO_2^- was detected and the NR-SOB-P did not produce N_2O. In the other case, 1.6 mM NO_2^- was detected and the NR-SOB-C did produce N_2O. The N_2O was detected in microcosms that had acetylene added to block the reduction of N_2O to N_2. Abiotic studies, described in the Appendix, showed that mineral medium containing sulfide turned yellow before turning pink in the presence of 5 and 10 mM NO_2^-. Jenneman et al. (1986) studied the effect of NO_3^- addition on sewage samples. They reported that color changes from "green or yellow-green to pink" occurred in medium and was followed by the appearance a white cloudy precipitate. These authors thought the precipitate was the result of oxidation products from the reducing agent cysteine. In another study, Jenneman et al. (1996) described enrichment cultures as turning from a transparent yellow color to a yellowish-white, cloudy suspension. Analysis of the particulate fraction showed it to be elemental sulfur and calcite crystals. There was no conclusive evidence that other forms of sulfur were present.

The appearance of a yellow color in mineral medium with sulfide has been observed in different studies. It would be interesting to determine if the yellow color is due to transient oxidation states of sulfide in the presence of nitrate-reducing activities in cultures and, or an abiotic reaction between nitrogen oxides and sulfur species. Nemati et al. (2001) suggested that increased corrosion rates could occur after nitrate amendment due to the formation of aggressive species of sulfur, such as polysulfide, elemental sulfur and thiosulfate, during sulfide oxidation.

Until recently, the detection of the sulfide oxidation products such as thiosulfate and polythionates was difficult because of poor resolution. Lately, methods such as capillary electrophoresis (Padarauskas et al. 2000) and ion-pair chromatography with a postcolumn azide-iodide reaction (Miura and Watanabe 2001) have been used. The possible production of sulfide-oxidation products in oil field waters should be investigated along with the suitability of the methods for oil field cultures.

7.2.5 Studies of anaerobic NR-SOB from oil reservoirs

Most of the work on sulfur bacteria has centered around aerobic sulfur bacteria and very little work has been done on anaerobic and mixotrophic NR-SOB. In this research project, three nitrate-amended oil fields were examined and three different types of anaerobic NR-SOB enrichment cultures were found. When Telang et al. (1999) looked at produced water from five oil fields in Canada and USA, they found that only the Coleville field in Saskatchewan contained NR-SOB that was represented on the reverse sample genome probe (RSGP) master filter. The Pembina oil field showed sulfide oxidation activity in CSB medium with the medium turning pink and the west Texas brines had slow sulfide removal with the CSB medium turning yellow (activity in CSB medium is described in Chapter 3 and Eckford and Fedorak 2002b). More moderate and high temperature oil fields should be examined for NR-SOB and methods should be developed so that these bacteria can be purified, preserved and studied.

7.2.6 Choosing the right NO_3^- formulation that would be suitable for nitrate amendment in oil reservoirs

From an economic perspective, two issues are important to oil production during nitrate amendment. One issue has to do with the type of NO_3^- that would be added to a reservoir. The nitrate-amendment study, described in this thesis, used KNO_3 as a source of NO_3^-. Hitzman and Sperl (1994) mention that "heterotrophic denitrifying bacteria in produced water of oil fields are uniformly able to produce cultures of high viscosity when starved for ammonia". They suggest that the use of a metallic salt of NO_3^- (sodium or potassium) causes cultures to become nitrogen starved and as a result, the excess carbon is transformed into viscous extracellular polysaccharides. Would the viscous extracellular polysaccharides be beneficial or detrimental to an oil field? Hitzman and Sperl (1994) also mention that if NH_4^+ in the form of NH_4NO_3 is added to flood waters, a mixed bacterial population containing many diverse types of microorganisms would be induced. How this induction would affect an oil field must be considered. The idea of inducing a mixed bacterial population introduces the second issue that is of importance for oil production during nitrate amendment. If NH_4NO_3 is added to the waterflood, the induction of bacteria could include those that can use alkylkbenzenes, alkanes or alkenes as substrates (Heider et al. 1999). The idea that hydrocarbons could be biodegraded during nitrate-amended waterflooding must be considered. Microcosm studies could be used to look at which form of NO_3^- is best for an oil field and if there would be significant hydrocarbon loss by induced bacteria. Most of the oil field waters described in Chapter 3 and Eckford and Fedorak (2002b) contained ammonium and Collins (1975) also mentioned that many oil field waters contain NH_4^+. In these cases, the type of NO_3^- to be used in the oil field may not matter. Of course, NH_4NO_3 can be explosive and this fact needs to be considered before it is used in an oil reservoir. Jenneman et al. (1999) mention that NH_4NO_3 was used successfully along with monobasic sodium phosphate for injection into the Coleville, Saskatchewan oil field.

7.2.7 The effects of nitrate-reduction products in oil reservoirs

One recurring issue for nitrate-amendment studies centers around the reduced forms of NO_3^- which are capable of raising the redox potential of an environment and inhibiting SRB. Studies have shown that NO_2^- (Reinsel et al. 1996, Myhr et al. 2002) and N_2O (Jenneman et al. 1986) are involved. The work described in the Appendix showed that NO_2^- or N_2O may be implicated in raising the redox potential of a microcosm and removing sulfide. Generally, under denitrifying conditions, if there is no limiting factor, nitrate reduction will proceed to N_2. If, for some reason, an electron donor (sulfide or an organic compound) or acceptor (NO_3^-) is limiting, or an enzyme in a denitrification step is inhibited, the denitrification process halts. In this case, an intermediate product of nitrate reduction may accumulate. This was shown in Chapter 6 where NO_2^- accumulated in microcosms with NR-SOB and low sulfide concentrations.

The nitrate-reduction products that could accumulate may be NO_2^-, NO or N_2O. As well, NRB that are not denitrifiers may produce only certain types of nitrate-reducing enzymes which could result in the accumulation of any of these products (Tiedje 1988). No study has shown whether these nitrate-reduction products would accumulate in oil reservoirs after nitrate amendment to the oil fields. As well, no study has shown whether NO could inhibit SRB activity in oil field settings. Nitric oxide is difficult to measure and would require sensitive gas analyzers and possibly a chemiluminescence NO-NO_2-NO_x analyzer (Kielemoes et al. 2000).

7.2.8 Molecular biology and cultural methods for DAP-NRB enumeration

The results for the DAP medium used for enumerating DAP-NRB in wastewater and oil field water samples showed MPN tubes at higher dilutions that were turbid with no measurable NH_4^+. It was thought that because the medium could grow bacteria other than DAP-NRB, the NH_4^+ produced by DAP-NRB may have been used by bacteria in the culture tubes. This would result in falsely low MPN results. One way to determine if DAP-NRB are in the end dilution MPN tubes is to use molecular biology methods. Michotey et al. (2000) obtained good enumeration results for environmental samples using MPN-polymerase chain reaction (PCR) and competitive PCR methods for enumerating denitrifying bacteria which contain the *nirS* gene encoding the cd_1 type Nir. One process preformed by DAP-NRB is called respiratory nitrite-ammonification and NH_4^+ is the end product. The reaction in DAP-NRB is catalyzed by cytochrome *c* nitrite reductase, a Nrf protein which is not present in denitrifying bacteria (Simon 2002). The turbid higher dilution MPN tubes that did not contain detectable NH_4^+ could be analyzed for cytochrome *c* nitrite reductase using molecular biology methods.

Culturing methods as described in section 5.4 using DAP medium, with and without NH_4^+, may help to determine how DAP medium can be used for environmental samples and to interpret MPN results using DAP medium. Bonin (1996) mentioned that the addition of NH_4^+ to DAP medium should inhibit assimilatory nitrate reduction. The idea is that NH_4^+, produced by DAP-NRB in MPN culture tubes with ammonium-containing DAP medium, would not be removed by other bacteria in the sample. Comparison could be made of the results from two MPN enumeration procedures for DAP-NRB in an environmental sample using DAP medium with and without NH_4^+. Two additional MPN enumerations for DAP-NRB could be compared. These enumerations would be done after nitrate amendment to the environmental sample. The nitrate amendment would be as described in Chapter 4 and Eckford and Fedorak (2002c) and the MPN procedures would be done using DAP medium with and without NH_4^+.

For MPN enumerations using ammonium-containing DAP medium to be valid, preliminary evaluations need to be done. Tests would need to be performed so that NH_4^+ analysis by the indophenol blue spot test could be normalized to an established threshold level that accounts for any NH_4^+ in the ammonium-containing DAP medium not assimilated by bacteria. As well, two matters need to be considered for the enumeration of DAP-NRB using ammonium-containing DAP medium. First, NH_4^+ added to the DAP medium should be at a concentration that would be removed by bacteria in the MPN culture tubes. Second, if the NH_4^+ in the medium is not removed by the bacteria in the sample, then the total NH_4^+ in a turbid MPN tube must exceed the threshold NH_4^+ level to confirm that NH_4^+ was produced by DAP-NRB in that tube.

7.2.9 Studies of biofilm bacteria from various oil reservoirs

No study on oil field bacteria can be complete without the mention of biofilms. The work presented in this research project has centered on planktonic bacteria in oil field waters. Chapter 1, Table 1.3, describes five studies where cores and columns have been used to study the control of sulfide production using NO_3^-. These studies have been used as indications for the effective use of nitrate amendment in sulfide-containing oil fields. Clearly, laboratory microcosm studies using oil field waters are a quick and economical means to determine if an oil field is amenable for NO_3^- treatment. This research project has shown that three different types of planktonic NR-SOB were isolated from three different oil fields. In order to understand the planktonic and biofilm dynamics of these bacteria in nitrate-amendment conditions, it would be of value to use cores or columns consisting of specific oil field formation material and bacteria specific to each oil field. Electron microscopy along with culture and molecular biology techniques could be used to study the bacterial associations within biofilms for oil fields under investigation. A realistic understanding of microbial activity in oil fields will not be attained without this type of work.

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APPENDIX

Abiotic changes in anaerobic mineral medium with acetate, benzoate, nitrate, nitrite and sulfide

A.1 Introduction

Nitrate-reducing bacteria (NRB) that reduce NO_3^- and produce NO_2^- and, or N_2O are considered important for the inhibition of sulfate-reducing bacteria (SRB). SRB reduce $SO_4^=$ and produce sulfide which contaminates their surroundings. The NO_2^- and N_2O, created under biotic conditions, are thought to raise the redox potential of an environment, thereby preventing SRB from producing sulfide (Jenneman et al. 1986a, Reinsel et al. 1996). A simple anaerobic experiment was done to see if, under abiotic conditions, NO_2^- or N_2O would influence sulfide removal.

A.1.1 Test conditions

The experiment was conducted with clean, scratch-free 16 x 125 mm Hungate type anaerobic culture tubes (Bellco Glass Inc., Vineland, NJ) that could be used to determine absorbance readings with a Spectronic 20 spectrophotometer. The sample holder was modified and was fitted with a device that excluded light during absorbance readings on medium in Hungate tubes.

CSB(2) medium as described in Chapter 6 was used. When NO_3^-, NO_2^- and sulfide were included in the medium, they were made from KNO_3, $NaNO_2$ and $Na_2S{\cdot}9H_2O$, respectively. The medium was prepared anaerobically, as described in Chapter 2 and Eckford and Fedorak (2002), and was used in three experiments. The final volume of medium in each Hungate tube was 10 mL.

Two abiotic experiments were done with CSB(2) medium and NO_2^-. They were: CSB(2) medium and NO_2^- [referred to as CSB(2) NO_2^-] and CSB(2) medium and NO_2^- plus 20 mM NO_3^- [referred to as CSB(2) NO_2^- plus NO_3^-]. One experiment was done with CSB(2) medium and N_2O plus 20 mM NO_3^- [referred to as CSB(2) N_2O]. The CSB(2) medium contained a vitamin solution (section 5.2.2), 30 mM sodium acetate and 1.4 mM sodium benzoate. For the abiotic NO_2^- experiment, about 2 mM sulfide was added to the medium. Nitrite was added anaerobically from a sterile, anaerobic 0.1 M stock solution to give concentrations of 10, 5, 2, 1 and 0.5 mM for each set of experiments. The CSB(2) medium for the abiotic N_2O experiment contained 0.8 mM sulfide with 0.04, 0.02, 0.009 and 0.004 mmol of filtered N_2O gas (Praxair, Mississauga, ON, Canada). A syringe was used to add N_2O to the Hungate tubes. The syringe was flushed several times with N_2O before being used for N_2O transfers to the tubes. Sterile medium control tubes were prepared with no NO_2^- or N_2O added to CSB(2) medium. All tubes were kept at 30°C in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI).

Absorbance readings were taken at 420 nm with the medium control tubes used as blanks. Absorbance readings from 350 to 440 nm were taken of CSB(2) medium that had turned yellow. The absorbance readings were compared. The 420 nm wavelength gave a sufficiently high absorbance reading and was chosen for the experiment. The tubes were not shaken before the absorbance readings were taken, in order to prevent interference from any precipitate in the tubes. Sulfide concentrations were measured using the CHEMetrics (Calverton, VA) methylene blue method.

A.2 Results

For the CSB(2) NO_2^- and CSB(2) NO_2^- plus NO_3^- experiments, all the tubes had various shades of yellow by day 2. Absorbance readings were taken for the CSB(2) NO_2^- plus NO_3^- experiment on days 4 to 14. The results are shown in Figure A.1a. Absorbance readings were taken for the CSB(2) NO_2^- experiment on days 3 to 12. The results are shown in Figure A.1b. At no time did the medium control tubes turn color. For both experiments (Figures A.1a and A.1b), the medium in the tubes was colorless on day 0. The yellow color that formed in the medium on day 2 increased in intensity for the tubes with 5 and 10 mM NO_2^- and then decreased until a pink color was seen. When the pink color appeared in the medium and sulfide analysis was done, the sulfide concentration was below detection in the tubes. There was a linear relationship between the 420 nm absorbance readings based on the yellow color in the medium and the initial NO_2^-

concentrations in the tubes. This is shown in Figure A.2 for the day 4 results using CSB(2) NO_2^- plus NO_3^- tubes.

The observations for the tubes from both abiotic NO_2^- experiments were similar, and only the observations for the CSB(2) NO_2^- plus NO_3^- experiment are given in Table A.1. A transient white precipitate was observed in the tubes. On day 42, the results for both experiments were the same as day 24.

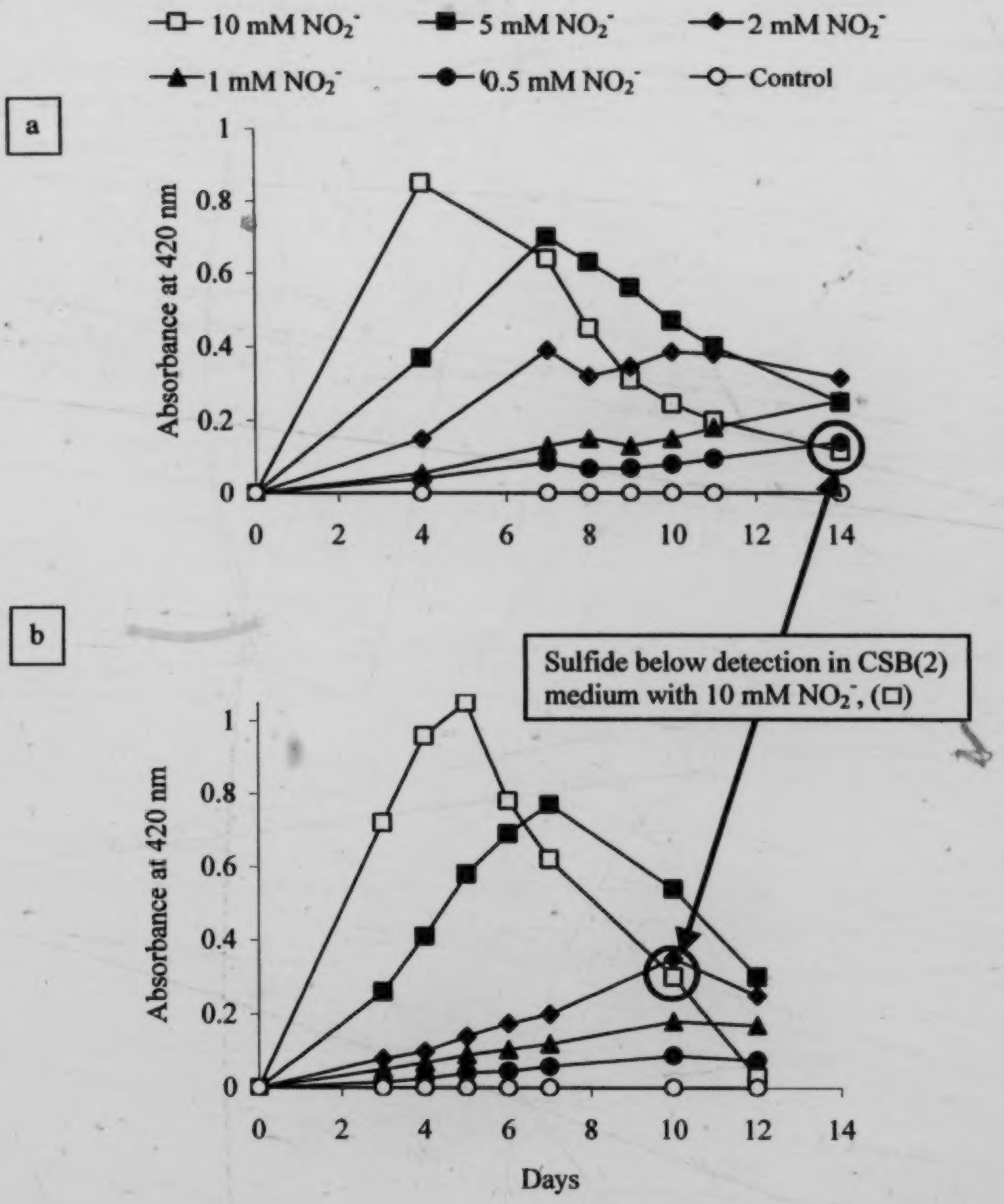
For the CSB(2) NO_2^- plus NO_3^- experiment, sulfide analysis was done on days 0, 7 and 14. The sulfide results are shown in Figure A.3. By day 14, the medium in the tube that initially contained 10 mM NO_2^- was slightly pink and the sulfide concentration was below detection. On day 16, the medium in the tube was very pink (Table A.1). Only the medium in the tubes with 5 and 10 mM NO_2^- became pink and had a white precipitate.

For the CSB(2) NO_2^- experiment, by day 10, the sulfide concentration in the tube that initially contained 10 mM NO_2^- was below detection and the medium in the tube was very slightly pink. On day 12, the medium was very pink. On day 20, the medium in the tube that initially contained 5 mM NO_2^- was very pink. No sulfide measurement was done on this tube.

For the CSB(2) N_2O experiment, the medium in the tube with 0.04 mmol N_2O (the highest amount added) turned pink in 11 d and no sulfide remained. No yellow color was observed in the medium for any of the tubes. The medium in the tubes with 0.02, 0.009 and 0.004 mmol N_2O remained clear and colorless for 6 weeks.

A.3 Conclusions

This experiment demonstrated that when certain concentrations of NO_2^- were present in CSB(2) mineral medium containing sulfide, the intensity of a yellow color progressively increased and then decreased in the liquid medium as sulfide was removed. As sulfide was removed, the changes in the medium from colorless to yellow to pink were likely due to an increase in the redox potential state and oxidation of sulfide. The redox indicator, resazurin, changed from the colorless reduced state to the pink oxidized state (Twigg 1945). These changes were not seen in sulfide-reduced mineral medium



Sulfide below detection in CSB(2) medium with 10 mM NO$_2^-$, (□)

Figure A.1 Color changes in medium measured by absorbance at 420 nm for abiotic experiment using CSB(2) NO$_2^-$ plus NO$_3^-$ (a) and CSB(2) NO$_2^-$ (b). Sulfide was below detection in the CSB(2) medium with 10 mM NO$_2^-$, as indicated. CSB(2) medium with no NO$_2^-$ was used as the blank. All the CSB(2) medium tubes with NO$_2^-$ were colorless on day 0.

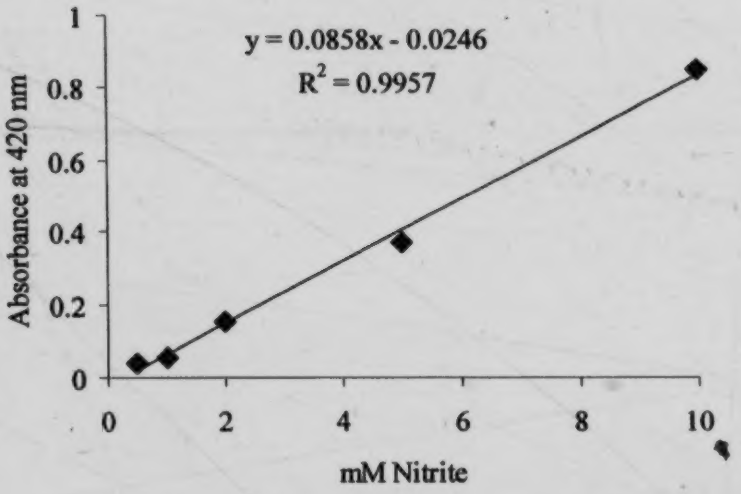


Figure A.2 Day 4 absorbance readings at 420 nm for abiotic experiment using CSB(2) medium with NO_2^- plus NO_3^-

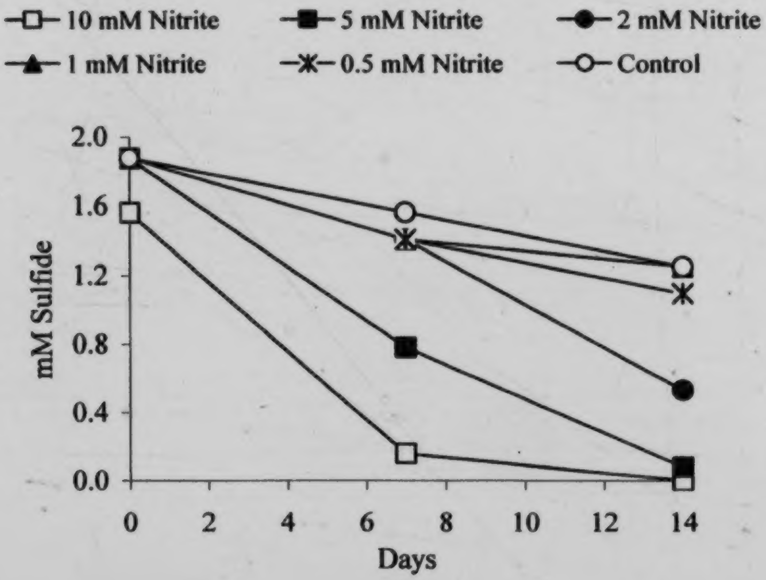


Figure A.3 Sulfide concentrations for abiotic experiment using CSB(2) medium with NO_2^- plus NO_3^-

Table A.1 Observed changes in CSB(2) medium with NO_2^- plus NO_3^- for abiotic experiment

Incubation time (d)	NO_2^- concentrations (mM)		
	10	5	≤2
0	Colorless	Colorless	Colorless
2	Yellow	Yellow	Yellow
10	Yellow with white precipitate	Yellow	Yellow
14	Slightly pink with white precipitate	Yellow	Yellow
16	Pink with white precipitate	Slightly pink with white precipitate	Yellow
24	Pink with no precipitate	Pink with no precipitate	Yellow

with only NO_3^-. The absorbance results shown in Figures A.1a and A.1b indicate that the CSB(2) NO_2^- plus NO_3^- and CSB(2) NO_2^- had similar color changes. The difference between the two tests was that sulfide was removed and the pink color change in the medium was observed sooner in the CSB(2) NO_2^- medium. It appears as though NO_3^- may have had an affect on the reaction between sulfide and NO_2^-. However, it should be noted that this experiment was done only once.

The yellow color that appeared in the presence of NO_2^- may have been caused by the presence of transient sulfide-oxidation products and the white precipitate could have been elemental sulfur as determined by Jenneman et al. (1996). The medium did not become yellow in the presence of N_2O, even though 0.04 mmol N_2O in the tube caused the redox indicator to turn pink.

Tiedje (1982) suggested that a rough indication for the presence of denitrifying bacteria (DNB) in nutrient broth-nitrate medium would be the conversion of at least 20% of the 5 mM NO_3^--N to N_2O. This conversion would produce about 0.005 mmol N_2O in the culture tube. If 100% of the 5 mM NO_3^- was converted to N_2O, then 0.02 mmol N_2O

would be in the tube. The 0.02 mmol N_2O is a lower concentration than the 0.04 mmol that was required to produce a pink color change in CSB(2) medium for the abiotic experiment. The CSB(2) medium tube with 0.02 mmol N_2O did not produce a pink color in this experiment.

Jenneman et al. (1986b) designed a method to detect microorganisms that produce gaseous nitrogen oxides. In this method, bacteria that produce NO or N_2O were detected by their ability to oxidize resazurin to a pink color. These authors concluded that the medium was oxidized by the presence of NO and N_2O. Unfortunately, for the experiment described here, there was no measurement of NO_2^- or N_2O to determine loss in the medium.

The use of nitrite to remove sulfide was the basis for a patent application by Burnes and Bhatia (1985). In this process, H_2S was removed from gas mixtures containing hydrocarbons when the gas mixture was treated with an aqueous solution of a water soluble nitrite at pH ≥ 5. The inventors claimed that as the treatment of the gas stream proceeded, "elemental sulfur is often formed so that the aqueous medium, while it may initially be a true solution, becomes a slurry containing the soluble, unreacted nitrite, various oxidation products of the hydrogen sulfide and particulate, elemental sulfur".

Kohl and Nielsen (1997) described a Sulfa-Check process, developed by NL Treating Chemicals/NL Industries, Inc. and marketed by Exxon Chemical Co., which was based on the use of a buffered aqueous solution of $NaNO_2$ to absorb and destroy sulfide. In this process, the initial charge of Sulfa-Check solution was gradually converted into a slurry as the solution absorbed H_2S. The slurry contained particles of elemental sulfur and other precipitated solids. These authors mentioned that the chemistry of the Sulfa-Check process was quite complex and the overall reaction was represented by the following equation:

$$3H_2S + NaNO_2 = NH_3 + 3S + NaOH + H_2O + \text{some } NO_x \qquad \text{(A.1)}$$

When the NaOH was in the presence of CO_2, sodium carbonate and bicarbonate were formed. Components of the spent slurry were given. The components included tetrathionate and sulfate in the liquid phase and sulfur, sodium sulfate and sodium tetrathionate in the solid phase. The NO_x was NO and when CO_2 concentrations were low (<0.1%), the release of NH_3 into the product gas was enhanced.

During microcosm studies described in Chapter 6, nitrate-reducing, sulfide-oxidizing (NR-SOB) enrichment cultures from oil fields P and N did not produce N_2O. These bacteria were incubated in single culture microcosms with CSB(2) medium containing 10 mM NO_3^-, acetate, benzoate and 0.8 mM sulfide. By day 1, the NR-SOB enrichment culture from oil field P produced 0.8 mM NO_2^-, the sulfide concentration was below detection and the medium in the microcosm had changed from colorless to pink. By day 3, the NR-SOB enrichment culture from oil field N produced 0.5 mM NO_2^-, the sulfide concentration had decreased 63% and the medium in the microcosm had changed from colorless to yellow. With each of these enrichment cultures, NO_2^- accumulated in the microcosms.

The results from the abiotic experiment, described here, and from the biotic microcosm studies, described in Chapter 6, indicate that sulfide can be removed from medium in the presence of NO_2^- and NR-SOB, respectively. For the abiotic experiment, it appeared as though NO_2^- reacted with and removed sulfide in CSB(2) medium. The tubes with CSB(2) medium and no NO_2^- and CSB(2) medium with only NO_3^- showed no color change. In the biotic experiments (Chapter 6), NR-SOB enrichment cultures removed sulfide in CSB(2) medium that initially contained only NO_3^-. When a pink color change was observed for the enrichment cultures, NO_2^- was present and likely raised the redox potential of the medium. When a yellow color was seen, NO_2^- was present and some sulfide remained in the medium.

It appears from these results that the removal of sulfide from liquid mineral medium in biotic conditions with NR-SOB occurred faster (1 d) with lower concentrations of NO_2^- raising the redox potential (<1mM). The results from the abiotic experiments indicate that a higher concentration of NO_2^- (10 mM) and a longer time (10 to 14 d) were required to remove sulfide and raise the redox potential of liquid mineral medium. Reinsel et al. (1996) mentioned in their work using NO_3^- and NO_2^- to control sulfide production in a sandstone column incubated at 60°C, "it is unclear why approximately 50% more nitrite (0.86 mM *vs* 0.57 mm) was required when nitrite was added directly to the North Sea column instead of being biologically produced".

A.4 Literature cited

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